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(54) Title: *TIE-2*, A NOVEL RECEPTOR TYROSINE KINASE

(57) Abstract

The present invention relates to a novel receptor tyrosine kinase, herein referred to as *tie-2*, to nucleotide sequences and expression vectors encoding *tie-2*, and to methods of inhibiting *tie-2* activity. The invention also relates to other members of the *tie-2* receptor tyrosine kinase family. Genetically engineered host cells that express *tie-2* may be used to evaluate and screen drugs involved in *tie-2* activation and regulation. The invention relates to the use of such drugs as agonists or antagonists of *tie-2* activity.

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TIE-2, A NOVEL RECEPTOR TYROSINE KINASE

1. INTRODUCTION

The present invention relates to a novel receptor tyrosine kinase, herein referred to as *tie*-2, to nucleotide sequences and expression vectors encoding *tie*-2, and to methods of inhibiting *tie*-2 activity. The invention also relates to other members of the *tie*-2 receptor tyrosine kinase family. Genetically engineered host cells that express *tie*-2 may be used to evaluate and screen drugs involved in *tie*-2 activation and regulation. The invention relates to the use of such drugs as agonists or antagonists of *tie*-2 activity.

2. BACKGROUND

Receptor tyrosine kinases comprise a large and evolutionarily conserved family of proteins comprised of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Receptor tyrosine kinases are involved in a variety of critical cellular processes such as cellular differentiation and proliferation. The binding of ligand to receptor tyrosine kinases induces the formation of receptor dimers followed by activation of receptor tyrosine kinase activity. This in turn results in phosphorylation of a number of intracellular substrates leading to a cascade of events eventually resulting in cellular responses such as changes in gene expression, cell morphology and cell proliferation.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play an important role in a variety of physiological processes such as embryonic development, wound healing and organ

regeneration. In particular, endothelial cell proliferation plays an important role during the development of the vascular system. In *in vitro* systems of angiogenesis, molecules that regulate tyrosine kinase activity have been found to modulate the invasion and tube formation of endothelial cells indicating that tyrosine phosphorylation plays a critical role in the regulation of endothelial cell proliferation and morphogenesis.

The cloning and characterization of two novel tyrosine kinases, expressed in endothelial cells, has recently been reported in the literature. Partanean et al. (1992, Mol. Cell. Biol. 12:1698-1707) identified a receptor tyrosine kinase, referred to as *tie*, which has 76% amino acid sequence homology with *tie*-2 in the cytoplasmic domain. The sequence homology between the two proteins diverges and is less pronounced in the extracellular and transmembrane domain (33% and 37% respectively). In addition, Dumont et al. (1992, Oncogene 7:1471-1480) reported the cloning and characterization of a partial cDNA clone encoding only the cytoplasmic domain of a novel receptor tyrosine kinase. The cloning of a full length representative of this particular clone, referred to as tek, has recently been reported and the deduced amino acid sequence of the tek tyrosine kinase receptor indicates broad homology with *tie*-2 (Ziegler et al., 1993, Oncongene 8:663-670).

The identification of novel tyrosine kinase receptors meditating physiological processes such as vasculogenesis and angiogenesis will lead to a more complete understanding of the molecular mechanisms controlling blood vessel formation. In addition, the identification of novel receptors involved in these processes will provide targets for development of therapeutic applications designed to treat disorders associated with aberrant blood vessel formation.

3. SUMMARY OF THE INVENTION

The present invention relates to a novel receptor tyrosine kinase receptor, herein referred to as *tie*-2, to nucleotide sequences and expression vectors encoding *tie*-2, and to methods of inhibiting *tie*-2 activity. The invention is based, in part, on the isolation of a cDNA clone, from a brain capillary cDNA library, encoding the *tie*-2 receptor tyrosine kinase.

The invention also relates to novel members of the *tie*-2 family of receptor tyrosine kinases. More specifically, the invention relates to members of the *tie*-2 family of receptor tyrosine kinases that are defined, herein, as those receptors demonstrating 80% homology at the amino acid level in substantial stretches of DNA sequence with *tie*-2. In addition, members of the *tie*-2 family of receptor tyrosine kinases are defined as those receptors containing an intracellular tyrosine kinase domain and, in the extracellular region of the protein, EGF-like repeats flanked by a single amino terminal immunoglobulin (Ig) domain and a carboxyl-terminal triplet of fibronectin (Fn) type III domains.

Northern blot analysis and in situ hybridization indicates that *tie*-2 is expressed in endothelial cell precursors (angioblasts) and in endothelial cells of the sprouting blood vessels throughout development and in all organs and tissues so far examined. Pharmaceutical reagents designed to modulate *tie*-2 activity may be useful for treating diseases and/or processes associated with angiogenesis and vasculogenesis.

The invention relates to expression systems designed to produce *tie*-2 receptor and/or cell lines expressing *tie*-2 receptor. For example, engineered cell lines expressing *tie*-2 on their surface may be advantageously used to identify *tie*-2 ligands and to screen and identify agonist and antagonist of the *tie*-

2 receptor. Additionally, expression of soluble recombinant *tie*-2 may be used to generate antibodi s against specific epitopes of the *tie*-2 protein and/or to screen peptide libraries for molecules that bind *tie*-2.

4. BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A, 1B and 1C. Nucleotide Sequence of murine *tie*-2. The AUG START codon and AGA STOP codon are underlined.

Fig. 2A. Deduced amino acid sequence and structure of *tie*-2. Amino acid sequence of *tie*-2 in single letter code. The potential signal sequence cleavage site is indicated by an arrowhead. Black dots mark the two cysteine residues, possible involved in sulfhydryl bonding of the immunoglobulin domain. The three EGF-like repeats are boxed. The three fibronectin type III domains are underlined. The transmembrane region is given in bold face letters. The tyrosine kinase domain is indicated by shaded boxes. The RGD triplet is marked by an asterisk.

Fig. 2B. Schematic diagram of the structure of *tie*-2. Ig. immunoglobulin domain; EGF, EGF-like repeats; FN, fibronectin type III domains; Kinase, tyrosine kinase domain; KI, kinase insertion.

Fig. 3. Amino acid sequence comparison of *tek*, *TEK* and *tie* with *tie*-2. Amino acid residues identical to *tie*-2 are represented by (-). Gaps are indicated by (.).

Fig. 4. Northern analysis of *tie*-2 expression in brain capillaries and total brain tissue from postnatal day 4 (P4) mice. A single transcript of approximately 4.7 kb, highly enriched in the capillary fraction was detected.

Fig. 5. *tie*-2 expression during brain development. Sagittal sections of adult brain (A,B), postnatal day 4 brain (C,D,G,H) and embryonic day 12.5

brain (E,F) were hybridized with a *tie*-2 probe. Bright-field (A,C,E) and corresponding dark-field micrographs (B,D,F) Arrowheads indicate expression in capillaries and arrows indicate hybridization signals over meningeal blood vessels. Higher magnification of a capillary sprout at the dorsal surface of a postnatal day 4 telencephalon (G). Higher magnification of a capillary in a deeper layer of the brain (H). No other than the vascular elements of the brain were found to synthesize *tie*-2 mRNA. LM, leptomeninges; LV, lateral ventricle; TE, telencephalon; (A,B,C,D,E,F) bar represents 110 μm; (G,H) bar represents 10 μm;

Fig. 6. Colocalization of *tie*-2 expression with immunostaining for PECAM (CD31). Adjacent section of E12.5 embryos were hybridize with a *tie*-2 probe (A) or stained with antibody recognizing PECAM (CD31)(B). Arrowheads indicate expression of *tie*-2 in the endothelial cell layer of a medium-sized blood vessel from the head region. Bar represents 10 μm.

Fig. 7. *Tie*-2 expression in organs and tissues of E 12.5 embryos and colocalization with immunostaining for PECAM (CD31). Adjacent sections of E 12.5 embryos were stained with an antibody recognizing PECAM (CD31) (A,C,E,G) or hybridized with a *tie*-2 probe (B,D,F,H). The expression pattern of *tie*-2 was found to be identical to the staining pattern fort PECAM (CD31). TE, telencephalon; LV, lateral ventricle; CP, choroid plexus of the lateral ventricle; DA, dorsal aorta; SC, spinal cord; EN, endocardium; MY, myocardium; BR, bronchus; SO, somite; Bar represents 110 μm.

Fig. 8. *Tie*-2 expression at embryonic day 8.5. Transverse section of an E 8.5 embryo and adjacent yolk sac. Hybridization signals were detectable in the endocardium, dorsal aorta, cardinal vein and the mesodermal (inner layer) of the yolk sac (A,B).

Arrowheads indicate hybridization signal over the marginal cells of an advanced stage blood island (C,D). Note the absence of hybridization signals in the neuroectoderm. CV, cardinal vein (head vein); BI, blood island; DA, dorsal aorta; EN, endocardial tissue; TE, telencephalon; YS, yolk sac. Bar, represents 50 μm.

5. DETAILED DESCRIPTION

The present invention relates to a novel receptor tyrosine kinases referred to herein as *tie*-2. The invention is based, in part, on the isolation of a cDNA clone encoding the *tie*-2 receptor tyrosine kinase from a brain capillary cDNA library. The invention also relates to novel members of the *tie*-2 receptor tyrosine kinase family as defined herein.

Results from Northern Blot analysis and *in situ* hybridization indicates that *tie*-2 is expressed specifically in the endothelial cell lineage. *Tie*-2 transcripts could be detected in endothelial cell precursors (angioblasts) and in endothelial cells of sprouting blood vessels throughout development and in all organs and tissues so far examined.

The invention relates to expression of the *tie*-2 receptor and/or cell lines that express the *tie*-2 receptor which may be used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of the *tie*-2 receptor. For example, anti-*tie*-2 antibodies may be used to inhibit *tie*-2 function. Alternatively, screening of peptide libraries with recombinantly expressed soluble *tie*-2 protein or cell lines expressing *tie*-2 protein may be useful for identification of therapeutic molecules that function by regulating the biological activity of *tie*-2.

For clarity of discussion, the invention is described in the subsections below by way of example

for the murine *tie*-2. However, the principles may be analogously applied to clone and express the *tie*-2 receptor of other species including humans.

5.1. THE *TIE*-2 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the *tie*-2 gene is depicted in Figures 1A, 1B, 1C (SEQ. ID NO: __) and Figure 2A (SEQ. ID NO: __), respectively. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the *tie*-2 gene product can be used to generate recombinant molecules which direct the expression of *tie*-2. In additional embodiments of the invention, nucleotide sequences which selectively hybridize to the *tie*-2 nucleotide sequence shown in FIG. 1 (SEQ ID NO: __) may also be used to express gene products with *tie*-2 activity. Hereinafter all such variants of the *tie*-2 nucleotide sequence will be referred to as the *tie*-2 DNA sequence.

In a specific embodiment described herein, the *tie*-2 gene was isolated by performing a polymerase chain reaction (PCR) in combination with two degenerate oligonucleotide primer pools that were designed from conserved protein regions of tyrosine kinases. As a template cDNA synthesized by reverse transcription of purified mRNA from capillaries of pooled P4-P8 mice brains was used. Gel-purified reaction products of the expected size were radiolabelled and used directly to screen a cDNA library constructed from the remainder of the capillary mRNA. Sequence analysis of the inserts from positive phages revealed that 13 of the cDNAs were derived from one mRNA species. The longest of these 13 clones, a fragment of 4640 bp, was sequenced completely. It contained a long open reading frame encoding a protein of 1123 amino acid residues.

Within the extracellular part, the FASTA program detected homologies to proteins including TAN-1 (Ellisen et al., 1991 Cell 66:649-661), Xotch (Coffman et al., 1990), Laminin (Sasaki et al., 1988 J. Biol. Chem. 263:16536-16544). Delta (Vässin et al., 1987 EMBO J. 6:3431-3440) and Perlecan (Noonan et al., 1991 J. Biol. Chem. 266:22939-22947) (around 30% sequence identity). The structural basis for these homologies are three EGF-like repeats in the center of the extracellular portion of *tie*-2 (Fig. 2A). EGF-like repeats consist of 30 to 40 amino acid residues often found in the extracellular parts of membrane-bound proteins or secreted proteins (Davies, 1990 New Biol 5:410-419). A common feature of these domains are six conserved cysteine residues, which are known to be involved in disulfide bonds. The three EGF domains in *tie*-2 contain two additional cysteine residues at the carboxyl-terminal end of each repeat.

The central EGF-like repeats are flanked by two different structural motifs, a single amino-terminal immunoglobulin (Ig) domain and a carboxyl-terminal triplet of fibronectin (FN) type III domains. Although the Ig domain does not exhibit clear sequence similarities to other known proteins (except for *tie* and *TEK*), the Ig domain has the conserved features of a C2-set domain including typical residues surrounding the first cysteine residue and the canonical GxYxC found at the second cysteine residue (Williams and Barclay, 1988 Ann. Rev. Immunol. 6:381-405).

The three fibronectin type III (FN III) domains are most closely related to FN III repeats present in the protein-tyrosine phosphatases Delta (Krueger et al., 1990 EMBO J. 9:3241-3252), LAR (Streuli et al., 1988 J. Exp. Med. 168:1553-1562) and DLAR (Streuli et al., 1989 Proc. Natl. Acad. Sci USA 86:8698-8702) and to FN III repeats of the axonal glycoprotein TAG-1 (Furley et al., 1990 Cell 61:157-170) (around 20%

sequence identity). These domains have been described as units of approximately 90 amino acids containing hydrophobic residues at characteristic positions. In addition, a total of nine potential N-glycosylation sites are located in the extracellular part of tie-2. The invention also relates to tie-2 genes isolated from other species, including humans. Members of the tie-2 family are defined herein as those receptors containing an intracellular tyrosine kinase domain, and, in the extracellular domain, EGF-like repeats flanked by two different structural motifs, a single amino-terminal immunoglobulin (Ig) domain and a carboxyl-terminal triplet of fibronectin (FN) type III domains. Such receptors may demonstrate about 80% homology at the nucleotide level, and even 90% homology at the amino acid level in substantial stretches of DNA sequence.

To isolate the tie-2 gene from other species a bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a radioactively labeled fragment of the murine tie-2 clone. Alternatively the murine tie-2 sequence can be used to design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. A polymerase chain reaction (PCR) based strategy may be used to clone, for example, the human tie-2. Two pools of degenerate oligonucleotides, corresponding to conserved motifs within the murine tie-2 may be designed to serve as primers in a PCR reaction. Conserved motifs may include the tyrosine kinase domain, the EGF-like repeats, the immunoglobulin (Ig) domain or the fibronectin (Fn) type III domains. The template for the reaction is cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express human tie-2 such as blood capillaries or endothelial cells. The PCR product may

be subcloned and sequenced to insure that the amplified sequences represent the *tie*-2 sequences. The PCR fragment may be used to isolate a full length *tie*-2 cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.)

Northern Blot analysis and *in situ* hybridization indicated that *tie*-2 was expressed in blood vessels during brain angiogenesis. Figure 4 demonstrates the presence of a 4.7 Mb mRNA highly enriched in the capillary fraction. A single RNA species could also be detected in organs like the brain, kidney and heart.

In situ hybridization experiments indicated that *tie*-2 mRNA was exclusively synthesized in the vasculature of adult brain, P4 brain, E12.5 brain E8.5 neuroectoderm (FIG. 5A-H). In P4 brain, *tie*-2 expression was detected in capillaries that are about to invade the neural tissue as well as in vessels that have already reached deep layers of neuroectoderm. In addition to capillaries, meningeal blood vessels and the choroid plexus were also found to synthesize *tie*-2 mRNA at comparable levels (FIGS. 5, 7B). At E12.5 the overall expression pattern was virtually identical to that observed at P4, although the density of labelled structures in E12.5 brain was reduced (Fig. 5C,O). This observation correlates with the less extensive vascularization of the embryonic brain (Bär, 1980 Adv. Anat. Embryol Cell Biol. 59:1-62). No hybridization signals were observed in the neuroectoderm of E8.5

embryos, because at that stage this tissue is still avascular (Fig. 8). In the adult brain, tie-2 expression was detectable, although it seemed to be reduced when compared to postnatal or embryonic stages. Hybridization signals persisted over larger vessels especially those of the meninges (Fig. 5A,B). The synthesis of tie-2 mRNA in smaller vessels and capillaries was barely detectable.

To identify the cell type, expressing *tie*-2 mRNA, *in situ* hybridization and immunohistochemistry was performed. The pattern of *tie*-2 expression was compared with the staining of cells with a monoclonal antibody against the endothelial cell-specific adhesion molecule PECAM (CD31). Figure 6 demonstrates the overlap between cells stained by the PECAM antibody and those cells labeling with a tie-2 probe. Figure 7 presents a survey on the coexpression of tie-2 and PECAM in several organs and tissues and it is clear from the results that tie-2 is coexpressed with PECAM in several organs and tissues.

The possible role of tie-2 in early stages of vascular development was investigated by *in situ* hybridization studies with E8.5 sections. The probe detected tie-2 mRNA in the mesodermal component of the yolk sac (FIG. 8). This finding is of particular interest, because it is in the mesodermal component of the yolk sac that the first signs of blood vessel development are evident. Clusters of mesenchymal cells form the so-called blood islands. At the margin of these aggregates cells, the so-called angioblasts, adopt an endothelial-like phenotype, whereas in the center cells differentiate into embryonic hemoblasts. Fig. 8 demonstrates that the peripheral angioblasts synthesize high levels of tie-2 mRNA.

Within the embryo proper, expression was seen in the anlagen of the vascular system, e.g. in the developing endocardium, the dorsal aortae and the

cardinal veins (Fig. 8A,B). It is likely that these signals stem from intraembryonic endothelial cell precursors, which are present in these structures at that stage, tie-2 expression could also be detected in the allantois and in blood vessels of the maternal decidua.

5.2. EXPRESSION OF *TIE*-2 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS *TIE*-2

In accordance with the invention, *tie*-2 nucleotide sequences which encode *tie*-2, peptide fragments of *tie*-2, *tie*-2 fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of *tie*-2 protein or a functionally equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the *tie*-2 sequence may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the *tie*-2 protein. Such DNA sequences include those which are capable of hybridizing to the murine *tie*-2 sequence under selective conditions.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. These alterations would in all likelihood be in regions of *tie*-2 that do not constitute functionally conserved regions. In contrast, alterations, such as deletions,

additions or substitutions of nucleotide residues in functionally conserved *tie*-2 regions would be expected to result in a nonfunctional *tie*-2 receptor. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the *tie*-2 sequence, which result in a silent change thus producing a functionally equivalent *tie*-2. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. The DNA sequences of the invention may be engineered in order to alter the *tie*-2 coding sequence for a variety of ends including but not limited to alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. For example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When using such expression systems it may be preferable to alter the *tie*-2 coding sequence to eliminate any N-linked glycosylation site.

In another embodiment of the invention, the *tie*-2 or a modified *tie*-2 sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric *tie*-2 protein expressing a heterologous epitope that is recognized by a

commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the *tie*-2 sequence and the heterologous protein sequence, so that the *tie*-2 may be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of *tie*-2 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize the *tie*-2 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (e.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

In order to express a biologically active *tie*-2, the nucleotide sequence coding for *tie*-2, or a functional equivalent, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The *tie*-2 gene products as well as host cells or cell lines transfected or transformed with recombinant *tie*-2 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal)

that bind to the receptor, including those that competitively inhibit binding of *tie*-2 ligand and "neutralize" activity of *tie*-2 and the screening and selection of drugs that act via the *tie*-2 receptor; etc.

5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the *tie*-2 coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the *tie*-2 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the *tie*-2 coding sequence; yeast transformed with recombinant yeast expression vectors containing the *tie*-2 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the *tie*-2 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the *tie*-2 coding sequence; or animal cell systems.

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the *tie*-2 DNA, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the *tie*-2 expressed. For example, when large quantities of *tie*-2 are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the *tie*-2 coding sequence may be ligated into the vector in frame with the lacZ coding region so

that a hybrid *tie*-2/lacZ protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the *tie*-2 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J.

3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express *tie*-2 is an insect system. In one such system, Autographa californica nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The *tie*-2 coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the *tie*-2 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the *tie*-2 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus

genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing *tie*-2 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of inserted *tie*-2 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire *tie*-2 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the *tie*-2 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the *tie*-2 coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage)

of protein products may be important for the function of the protein. The presence of nine consensus N-glycosylation sites in the *tie*-2 extracellular domain indicate that proper modification may be important for *tie*-2 function. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the *tie*-2 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the *tie*-2 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the *tie*-2 on the cell surface. Such engineered cell lines are

particularly useful in screening for drugs that affect *tie*-2.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk$^-$, hgprt$^-$ or aprt$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.2.2. IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS THAT EXPRESS THE *TIE*-2

The host cells which contain the coding sequence and which express the biologically active gene product

may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of *tie*-2 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the *tie*-2 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the *tie*-2 coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the *tie*-2 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the *tie*-2 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *tie*-2 sequence under the control of the same or different promoter used to control the expression of the *tie*-2 coding sequence. Expression of the marker in response to induction or selection indicates expression of the *tie*-2 coding sequence.

In the third approach, transcriptional activity for the *tie*-2 coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the *tie*-2 coding sequence or particular portions thereof. Alternatively, total nucleic acids

of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the *tie*-2 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like.

5.3. USES OF THE *TIE*-2 RECEPTOR AND ENGINEERED CELL LINES

The uses of the *tie*-2 receptor and engineered cell lines, described in the subsections below, may be employed equally well for the *tie*-2 family of receptor tyrosine kinases.

In an embodiment of the invention, engineered cell lines which express the entire *tie*-2 coding region or its ligand binding domain may be utilized to screen and identify the natural ligand and/or ligand antagonists as well as agonists. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways.

5.3.1 IDENTIFICATION OF *TIE*-2 LIGAND USING ENGINEERED CELL LINES

Engineered cell lines that express the *tie*-2 receptor, may be advantageously used to identify and characterize the natural *tie*-2 ligand(s). For example, a genomic or cDNA library may be transfected into the engineered cell lines. Any cell that expresses and secretes the *tie*-2 ligand, due to the transfer of a DNA clone capable of encoding the *tie*-2 ligand, will stimulate the biological activity of the recombinantly expressed the *tie*-2 receptor. The resulting autocrine loop will lead to preferential proliferation of cells expressing *tie*-2 ligand and the

resulting cell colonies may be used to isolate the DNA encoding the *tie*-2 ligand.

The engineered cell lines may also be used to assay tissue or cell extracts for their ability to activate *tie*-2 receptor tyrosine kinase activity. Activation of *tie*-2 tyrosine kinase activity may be assayed using a variety of methods. For example, in cells overexpressing the *tie*-2 receptor, overal incorporation of labeled PO_4 into the cell may be measured after contacting cells with extracts. Alternatively, the *tie*-2 protein may be immunoprecipitated following stimulation with extracts, using anti-*tie*-2 antibodies, followed by Western blot analysis using anti-tyrosine phosphate antibodies to determine whether the immunoprecipitated *tie*-2 has been tyrosine phosphorylated.

Once an extract is identified which contains *tie*-2 ligand, various procedures and techniques known in the art which include but are not limited to chromatography (e.g., reverse phase liquid, gell permeation, liquid exchange, ion exchange, size exclusion, affinity chromatography), centrifugation, electrophoretic procedures, differential solubility, or other standard techniques may be used to purify *tie*-2 ligand.

In a preferred embodiment, affinity chromatography using recombinantly expressed *tie*-2 covalently attached to a column matrix may be used to purify *tie*-2 ligand. Alternatively, a recombinantly expressed hybrid protein comprised of the *tie*-2 extracellular domain fused to the immunoglobulin protein A binding domain may be used to purify *tie*-2 ligand. The fusion protein may be used to prepare a column matrix over which cell extracts may be added, or, the fusion protein may be used to immunoprecipitate the *tie*-2 ligand from cell extracts.

Once purified, the *tie*-2 ligand may be subjected to microsequencing, using techniques routinely used by those skilled in the art to determine the amino acid sequence of a protein. If the *tie*-2 ligand molecule is blocked at the amino terminus, the protein may be chemically cleaved or partially enzymatically digested to yield peptide fragments that may be purified and sequenced.

A mixture of degenerate oligonucleotide probes may be designed using the information derived from the protein sequencing of the purified *tie*-2 ligand. The oligonucleotides may be labeled and used directly to screen a cDNA library for clones containing inserts with sequence homology to the oligonucleotide sequences. Alternatively, the oligonucleotides may be used as primers in a polymerase chain reaction. The amplified DNA fragment may be labeled and used to screen a library for isolation of full length clones.

5.3.2. SCREENING OF PEPTIDE LIBRARY WITH *TIE*-2 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the *tie*-2 may be accomplished by screening a peptide library with recombinant soluble *tie*-2 protein. Methods for expression and purification of

tie-2 are described in Section 5.2.1 and may be used to express recombinant full length *tie*-2 or fragments of *tie*-2 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of *tie*-2 may be separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with *tie*-2, it is necessary to label or "tag" the *tie*-2 molecule. The *tie*-2 protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothyiocynate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to *tie*-2, may be performed using techniques that are routine in the art. Alternatively, *tie*-2 expression vectors may be engineered to express a chimeric *tie*-2 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

To screen a peptide library the "tagged" *tie*-2 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between *tie*-2 and peptide species within the library. The library is then washed to remove any unbound *tie*-2 protein. If *tie*-2 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-*tie*-2 complex changes color, and can be easily identified and isolated physically

under a dissecting microscope with a micromanipulator. If a fluorescent tagged *tie*-2 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric *tie*-2 protein expressing a heterologous epitope has been used, detection of the peptide/*tie*-2 complex may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble *tie*-2 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell membrane to be functional. Methods for generating cell lines expressing *tie*-2 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated with the random peptide library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

5.3.3. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced *tie*-2 receptor. Such

antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the ligand binding site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind *tie*-2 may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* cells expressing *tie*-2.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity *tie*-2 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diphtheria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate *tie*-2 expressing cells.

For the production of antibodies, various host animals may be immunized by injection with the *tie*-2 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to *tie*-2 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce *tie*-2-specific single chain antibodies.

Antibody fragments which contain specific binding sites of *tie*-2 may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to *tie*-2.

5.4. USES OF *TIE*-2 CODING SEQUENCE

The *tie*-2 coding sequence may be used for diagnostic purposes for detection of *tie*-2 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of *tie*-2. In addition, mutated forms of *tie*-2, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed *tie*-2.

5.4.1. USE OF *TIE*-2 CODING SEQUENCE IN DIAGNOSTICS AND THERAPEUTICS

The *tie*-2 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of *tie*-2. For example, the *tie*-2 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of *tie*-2 expression; e.g., Southern or Northern analysis, including *in situ* hybridization assays.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of *tie*-2 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the *tie*-2 nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered

hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of *tie*-2 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of

flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5.4.2. USE OF DOMINANT NEGATIVE *TIE*-2 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase activity. Defective receptors can function as dominant negative mutations by suppressing the activation and response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be engineered into recombinant viral vectors and used in gene therapy in individuals that inappropriately express *tie*-2.

In an embodiment of the invention, mutant forms of the *tie*-2 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion or missense mutants of *tie*-2 that retain the ability to form dimers with wild type *tie*-2 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type *tie*-2. For example, the cytoplasmic kinase domain of *tie*-2 may be deleted resulting in a truncated *tie*-2 molecule that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Recombinant viruses may be engineered to express dominant negative forms of *tie*-2 which may be used to inhibit the activity of the wild type endogenous *tie*-2. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of *tie*-2, such as cancers.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant *tie*-2 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct those recombinant viral vectors containing *tie*-2 coding sequence. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant *tie*-2 molecules can be reconstituted into liposomes for delivery to target cells.

6. EXAMPLES: CLONING AND CHARACTERIZATION OF THE *TIE*-2 RECEPTOR TYROSINE KINASE

6.1. MATERIALS AND METHODS

6.1.1. ANIMALS AND TISSUES

Balb/c mice were mated overnight and the morning of vaginal plug detection was defined as 0.5 day of gestation. Organs were removed, frozen immediately in liquid nitrogen and stored at -80°C. Capillary fragments from pooled P4-P8 mice brains were prepared according to Risau et al. (1990 J. Cell Biol. 110:1757-1766). For *in situ* hybridization and immunohistochemistry, whole embryos or organs were fixed for 12 hours in freshly prepared 4% paraformaldehyde in PBS at 4°C, rinsed for 24-48 hours in 0.5 M sucrose at PBS at 4°C, embedded in TissueTek (Miles) and stored frozen at -80°C.

6.1.2. RNA EXTRACTION AND ANALYSIS

Total cytoplasmic RNA was isolated according to the method of Chomczynksi and Sacchi (1987 Anal. Biochem. 162:156-159). Enrichment for poly(A)$^+$ containing fractions was achieved by oligo(dT) chromatography using push columns (Stratagene). Aliquots of poly(A)$^+$ RNA were electrophoresed in agarose gels containing 0.66 M formaldehyde and transferred to Zeta-Probe membrane (Bio Rad) in 20x SSPE. Hybridizations were performed overnight in 0.5 M sodium phosphate butter, 5% SDS, 1% BSA, pH 7.5 at 68°C with $1x10^6$ cts/minute/ml of probe, which had been labelled with ^{32}P-dCTP according to the protocol of a random-primed DNA-labeling kit (Bohringer Mannheim). Membranes were washed under high-stringency conditions at 68°C in 0.1XSSPE. 0.5% SDS and autoradiographed at -80°C on Fuji films. Poly(A)$^+$ RNA from brain capillary fragments was isolated using a QuickPrep Micro mRNA purification kit from Pharmacia.

6.1.3. PCR AND cDNA CLONING

Capillary poly(A)$^+$ RNA was reverse transcribed and aliquots of the cDNA were used as templates in PCR reactions. The following oligonucleotides were used as primers: 5'-CAC/TCGIGAC/TC/TTIGCIGCIA/CG-3',5'-AC/TICCIAA/CIC/GA/TCCAIACA/CTC-3' (I stands for inosine) in addition to the following premiss described by Wilks et al. (1989, DPNAS Proc. Natl. Acad. Sci. USA 86:1603-1607):

5'-CGGATCCACAGNGACCT-3' and
C TT

3'-CTGCAGACCAGGAAACCTTAAGG-5'
A C T

The amplification products were separated in acrylamide gels. Fragments of the expected size were purified, labeled and used as probes to screen a random hexanucleoride-primed cDNA library constructed

with a Time Saver cDNA Synthesis Kit (Pharmacia). cDNA fragments were subcloned into Bluescript vectors (Stratagene). Sequencing was done by using nested oligonucleotide primers in combination with a 373 DNA Sequencer (Applied Biosystems) and with the Convention Sequenase System (USB).

6.1.4. *IN SITU* HYBRIDIZATION

Preparation of tissue sections and *in situ* hybridizations with single-stranded DNA probes or single-stranded RNA probes was performed as described by Schnürch and Risau (1991, Development 111:1143-1154). The probe for all hybridization experiments was derived from a 1179 bp DNA fragment encoding a portion of the putative *tie*-2 protein from amino acid 419 to amino acid 812.

6.1.5. IMMUNOHISTOCHEMISTRY

Embedded embryos and whole organs were sectioned on a Leitz cryostat 8 μm sections were mounted on organosilane-treated slides, dried overnight under vacuum and stored desiccated at -80°C. Sections were brought to room temperature, rehydrated in PBS for 5 minutes and incubated in 0.1% H_2O_2 in methanol for 15 minutes. After washing three times in PBS for 5 minutes each, nonspecific antibody binding was blocked by application of 20% normal goat serum in PBS for 20 minutes. Sections were washed, incubated with a rat monoclonal antibody against mouse PECAM for one hour, washed again and then incubated with a viotinylated gout anti-rat IgG (Dianova) for one hours. Color development was performed with a Vectastain ABC Kit (Vector Laboratories) according to the vendor's protocol. Sections were slightly counterstained with toluene blue, dehydrated and mounted.

6.2. RESULTS

6.2.1. cDNA CLONING AND STRUCTURE OF THE *TIE*-2 PROTEIN

The approach for the isolation of receptor tyrosine kinases expressed in endothelial cells of sprouting blood vessels involved the following steps. First, mRNA was purified from capillaries of pooled P4-P8 mice brains. A portion of the mRNA was reverse transcribed and used in PCR reactions with degenerate primers deduced from conserved protein regions of tyrosine kinases. Gel-purified reaction products of the expected size were radiolabelled and used directly as hybridization probes to screen a cDNA library constructed from the remainder of the capillary mRNA.

Partial sequencing of the inserts from positive phages revealed that 13 of the cDNAs were derived from one mRNA species. The longest of these 13 cDNAs, a fragment of 4640 bp, was sequenced completely (FIG. 1A, 1B, 1C). It contained a long open reading frame encoding a protein of 1123 amino acid residues (FIG. 2A). This deduced polypeptide has all features of a receptor tyrosine kinase: an amino terminal signal sequence followed by a long extracellular domain, a single hydrophobic transmembrane region and a cytoplasmic portion that contains a tyrosine kinase domain (Fig. 2B).

A survey for homologous proteins revealed that the predicted protein is most closely related to the two recently identified tyrosine kinases *tie* and *tek* (Partanen et al., 1992 Mol. Cell. Biol. 12:1698-1707; Dumont et al., 1992 Oncogene 7:1471-1480). With the exception of two residues, the protein sequence published for *tek* is identical to the intracellular part of our polypeptide, from position 823 to the carboxyl-terminal residue 1123. Both an extracellular domain and a transmembrane region are missing in the *tek* polypeptide (Fig. 3). It is therefore possible

that *tek* represents a partial sequence or that the mRNA encoding *tek* is the result of an alternative splice event, which results in the production of a cytoplasmic tyrosine kinase. Comparison of our protein with *tie* reveals a high degree of similarity especially in the cytoplasmic part (76% sequence identify). In the extracellular domain and the transmembrane region, the similarity is less pronounced (33% and 37% respectively; Fig. 3). Ziegler et al. (1993 Oncogene 8:663-670) have also reported the cloning of a cDNA whose translation product is a receptor tyrosine kinase with an overall similarity of more than 90% when compared to *tie*-2 (Fig. 3). It is therefore likely that this protein, which they called *TEK*, is the human homolog of *tie*-2. The intracellular part of *tie*-2 is also related to the product of the human ret protooncogene and to FGF receptors (Takahashi et al., 1989 Oncogene 4:805-806; Partanen et al., 1991 EMBO J. 10:1347-1354; Stark et al., 1991 Development 113:641-651). The similarity to both is about 43%. The intracellular part can be divided into three characteristic regions: the juxtamembrane sequence, the catalytic domain and the cytoplasmic tail. The kinase domain, which is split by an 14 amino acid insertion contains the GxGxxG consensus sequence that is part of the ATP binding site. Typical tyrosine kinase motifs like HRDLAARN and DFGL are present.

Within the extracellular part, the FASTA program detected homologies to proteins including TAN-1 (Ellisen et al., 1991 Cell 66:649-661), Xotch (Coffman et al., 1990 Science 249:1438-1440), Laminin (Sasaki et al., 1988 J. Biol. Chem. 263:16536-16544). Delta (Vässin et al., 1987 EMBO J. 6:3431-3440) and Perlecan (Noonan et al., 1991 J. Biol. Chem. 266:22939-22947) (around 30% sequence identity). The structural basis for these homologies are three EGF-like repeats in the

center of the extracellular portion of *tie*-2 (Fig. 2A and 2B). EGF-like repeats consist of 30 to 40 amino acid residues often found in the extracellular parts of membrane-bound proteins or secreted proteins (Davies, 1990 New Biol. 5:410-419). A common feature of these domains are six conserved cysteine residues, which are known to be involved in disulfide bonds. The three EGF domains in *tie*-2 contain two additional cysteine residues at the carboxyl-terminal end of each repeat.

The central EGF-like repeats are flanked by two different structural motifs, a single amino-terminal immunoglobulin (Ig) domain and a carboxyl-terminal triplet of fibronectin (FN) type III domains. Although the Ig domain does not exhibit clear sequence similarities to other known proteins (except for *tie* and *TEK*), the Ig domain has the conserved features of a C2-set domain including typical residues surrounding the first cysteine reside and the canonical GxYxC found at the second cysteine residue (Williams and Barclay, 1988 Ann. Rev. Immunol. 6:381-405).

The three fibronectin type III (FN III) domains are most closely related to FN III repeats present in the protein-tyrosine phosphatases Delta (Krueger et al., 1990 EMBO J. 9:3241-3252), LAR (Streuli et al., 1988 J. Exp. Med. 168:1553-1562) and DLAR (Streuli et al., 1989) and to FN III repeats of the axonal glycoprotein TAG-1 (Furley et al., 1990 Cell 61:157-170) (around 20% sequence identity). These domains have been described as units of approximately 90 amino acids containing hydrophobic residues at characteristic positions. In addition, a total of nine potential N-glycosylation sites are located in the extracellular part of *tie*-2.

6.2.2. *TIE*-2 EXPRESSION IN BRAIN CAPILLARIES

The amount of *tie*-2 in mRNA from P4-P8 brain capillaries was compared with total P4 brain by Northern analysis. To avoid artifactual results, the part of the cDNA that encodes the least conserved regions of the protein, namely the three FN III domains, the transmembrane region and the juxtamembrane portion was used. Fig. 4 demonstrates the presence of a 4.7 kd mRNA highly enriched in the capillary fraction. RNA expression was also detected in organs like brain, kidney and heart.

6.2.3. *TIE*-2 EXPRESSION DURING BRAIN DEVELOPMENT

Investigation of the spatial and temporal expression profile of *tie*-2 during brain development was performed by in situ hybridization. When analyzed, it became evident that *tie*-2 mRNA is exclusively synthesized in the vasculature of (Fig. 5A-H) adult brain, P4 brain, E12.5 brain and E8.5 neuroectoderm. No other brain components were labelled. In P4 brain, *tie*-2 expression was detected in capillaries that are about to invade the neural tissue as well as in vessels that have already reached deeper layers of the neuroectoderm. High magnifications in Fig. 5G,H clearly show the high concentration of silver grains over the vascular perikarya. In addition to capillaries, meningeal blood vessels and the choroid plexi were also found to synthesize *tie*-2 mRNA at comparable levels (Figs 5, 7B). At 312.5 the overall expression pattern was virtually identical to that observed at P4, although the density of labelled structures in E12.5 brain was reduced (Fig. 5C,D). This observation correlates with the less extensive vascularization of the embrycnic brain (Bär, 1980 Adv. Anat. Embryol. Cell Biol 59:1-

62). No hybridization signals were observed in the neuroectoderm of E8.5 embryos, because at that stage this tissue is still avascular (Fig. 8). In the adult brain, *tie*-2 expression was detectable, although it seemed to be reduced when compared to postnatal or embryonic stages. Hybridization signals persisted over larger vessels especially those of the meninges (Fig. 5A,B). The synthesis of *tie*-2 mRNA in smaller vessels and capillaries was barely detectable.

6.2.4. ENDOTHELIAL CELL-SPECIFIC EXPRESSION OF *TIE*-2

To identify the cellular source of *tie*-2 mRNA, *in situ* hybridization and immunohistochemistry on adjacent sections were performed. The pattern of *tie*-2 hybridization signals was compared with the immunohistochemical staining of a monoclonal antibody against the endothelial cell-specific adhesion molecule PECAM (CD31) (Newman et al., 1990, Science 247:1219-1222). Fig. 6 shows a representative example of one such experiment. The antibody stains the continuous layer of endothelial cells surrounding the lumen of a medium-sized blood vessel in the head region of an E12.5 embryo. On the adjacent section, the *tie*-2-specific probe labels the vessel in an identical way. These results, together with the northern hybridization signal detected in RNA from a capillary fraction highly enriched for endothelial cells, provide strong evidence for endothelium-specific expression of *tie*-2 and PECAM in several organs and tissues. It is clear that *tie*-2 mRNA is present in endothelial cells all over the body. Strong hybridization signals were associated with the heart endocardium as well with the myocardial blood vessels (Fig. 7E,F). The same holds true for the endothelium of the dorsal aorta (Fig. 7C,D), the intersomitic vasculature, the vessels surrounding the

lung bronchia and the capillaries perforating the spinal cord (Fig. 7H,D). In summary, *tie*-2 gene expression seems to be a general feature of endothelial cells.

6.2.5. *TIE*-2 EXPRESSION DURING EARLY STAGES OF DEVELOPMENT

The possible role of *tie*-2 in early stages of vascular development was investigated by *in situ* hybridization studies with E8.5 sections. The probe detected *tie*-2 mRNA in the mesodermal component of the yolk sac (Fig. 8). This finding is of particular interest, because it is in the mesodermal component of the yolk sac that the first signs of blood vessel development are evident. Clusters of mesenchymal cells form the so-called blood islands. At the margin of these aggregates cells, the so-called angioblasts, adopt an endothelial-like phenotype, whereas in the center cells differentiate into embryonic hemoblasts. Fig. 8 demonstrates that the peripheral angioblasts synthesize high levels of *tie*-2 mRNA.

Within the embryo proper, expression was seen in the anlagen of the vascular system, e.g. in the developing endocardium, the dorsal aortae and the cardinal veins (Fig. 8A,B). It is likely that these signals stem from intraembryonic endothelial cell precursors, which are present in these structures at that stage, *tie*-2 expression could also be detected in the allantois and in blood vessels of the maternal decidua.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Risau, Werner
Schnurch, Harald

(ii) TITLE OF INVENTION: TIE-2 A NOVEL TYROSINE RECEPTOR KINASE

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/152,552
(B) FILING DATE: 12-NOV-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Coruzzi, Laura A.
(B) REGISTRATION NUMBER: 30,742
(C) REFERENCE/DOCKET NUMBER: 7683-059

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 790-9090
(B) TELEFAX: (212) 869-9741
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4640 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 341..3712

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 301 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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        275                 280                 285

Thr Tyr Ala Gly Ile Asp Cys Ser Ala Glu Glu Ala Ala
    290                 295                 300
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1124 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Asp Ser Leu Ala Ser Leu Val Leu Cys Gly Val Ser Leu Leu Leu
1               5                   10                  15

Ser Gly Thr Val Glu Gly Ala Met Asp Leu Ile Leu Ile Asn Ser Leu
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                20                  25                  30

Pro Leu Val Ser Asp Ala Glu Thr Ser Leu Thr Cys Ile Ala Ser Gly
        35                  40                  45

Trp Arg Pro His Glu Pro Ile Thr Ile Gly Arg Asp Phe Glu Ala Leu
    50                  55                  60

Met Asn Gln His Gln Asp Pro Leu Glu Val Thr Gln Asp Val Thr Arg
65                  70                  75                  80

Glu Trp Ala Lys Lys Val Val Trp Lys Arg Glu Lys Ala Ser Lys Ile
                85                  90                  95

Asn Gly Ala Tyr Phe Cys Glu Gly Arg Val Arg Gly Glu Ala Ile Arg
            100                 105                 110

Ile Arg Thr Met Lys Met Arg Gln Gln Ala Ser Phe Leu Pro Ala Thr
        115                 120                 125

Leu Thr Met Thr Val Asp Lys Gly Asp Asn Val Asn Ile Ser Phe Lys
    130                 135                 140

Lys Val Leu Ile Lys Glu Glu Asp Ala Val Ile Tyr Lys Asn Gly Ser
145                 150                 155                 160

Phe Ile His Ser Val Pro Arg His Glu Val Pro Asp Ile Leu Glu Val
                165                 170                 175

His Leu Pro His Ala Gln Pro Gln Asp Ala Gly Val Tyr Ser Ala Arg
            180                 185                 190

Tyr Ile Gly Gly Asn Leu Phe Thr Ser Ala Phe Thr Arg Leu Ile Val
        195                 200                 205

Arg Arg Cys Glu Ala Gln Lys Trp Gly Pro Glu Cys Asn His Leu Cys
    210                 215                 220

Thr Ala Cys Met Asn Asn Gly Val Cys His Glu Asp Thr Gly Glu Cys
225                 230                 235                 240

Ile Cys Pro Pro Gly Phe Met Gly Arg Thr Cys Glu Lys Ala Cys Glu
                245                 250                 255

Leu His Thr Phe Gly Arg Thr Cys Lys Glu Arg Cys Ser Gly Gln Glu
            260                 265                 270

Gly Cys Lys Ser Tyr Val Phe Cys Leu Pro Asp Pro Tyr Gly Cys Ser
        275                 280                 285

Cys Ala Thr Gly Trp Lys Gly Leu Gln Cys Asn Glu Ala Cys His Pro
    290                 295                 300

Gly Phe Tyr Gly Pro Asp Cys Lys Leu Arg Cys Ser Cys Asn Asn Gly
305                 310                 315                 320

Glu Met Cys Asp Arg Phe Gln Gly Cys Leu Cys Ser Pro Gly Trp Gln
                325                 330                 335

Gly Leu Gln Cys Glu Arg Glu Gly Ile Pro Arg Met Thr Pro Lys Ile
            340                 345                 350

Val Asp Leu Pro Asp His Ile Glu Val Asn Ser Gly Lys Phe Asn Pro
        355                 360                 365

Ile Cys Lys Ala Ser Gly Trp Pro Leu Pro Thr Asn Glu Glu Met Thr
    370                 375                 380
```

Leu Val Lys Pro Asp Gly Thr Val Leu His Pro Lys Asp Phe Asn His
385 390 395 400

Thr Asp His Phe Ser Val Ala Il Phe Thr Ile His Arg Ile Leu Pro
405 410 415

Pro Asp Ser Gly Val Trp Val Cys Ser Val Asn Thr Val Ala Gly Met
420 425 430

Val Glu Lys Pro Phe Asn Ile Ser Val Lys Val Leu Pro Lys Pro Leu
435 440 445

Asn Ala Pro Asn Val Ile Asp Thr Gly His Asn Phe Ala Val Ile Asn
450 455 460

Ile Ser Ser Glu Pro Tyr Phe Gly Asp Gly Prc Ile Lys Ser Lys Lys
465 470 475 480

Leu Leu Tyr Lys Pro Val Asn His Tyr Glu Ala Trp Gln His Ile Gln
485 490 495

Val Thr Asn Glu Ile Val Thr Leu Asn Tyr Leu Glu Pro Arg Thr Glu
500 505 510

Tyr Glu Leu Cys Val Gln Leu Val Arg Arg Gly Glu Gly Gly Glu Gly
515 520 525

His Pro Gly Pro Val Arg Arg Phe Thr Thr Ala Ser Ile Gly Leu Pro
530 535 540

Pro Pro Arg Gly Leu Asn Leu Leu Pro Lys Ser Gln Thr Thr Leu Asn
545 550 555 560

Leu Thr Trp Gln Pro Ile Phe Pro Ser Ser Glu Asp Asp Phe Tyr Val
565 570 575

Glu Val Glu Arg Arg Ser Val Gln Lys Ser Asp Gln Gln Asn Ile Lys
580 585 590

Val Pro Gly Asn Leu Thr Ser Val Leu Leu Asn Asn Leu His Pro Arg
595 600 605

Glu Gln Tyr Val Val Arg Ala Arg Val Asn Thr Lys Ala Gln Gly Glu
610 615 620

Trp Ser Glu Asp Leu Thr Ala Trp Thr Leu Ser Asp Ile Leu Pro Pro
625 630 635 640

Gln Pro Glu Asn Ile Lys Ile Ser Asn Ile Thr His Ser Ser Ala Val
645 650 655

Ile Ser Trp Thr Ile Leu Asp Gly Tyr Ser Ile Ser Ser Ile Thr Ile
660 665 670

Arg Tyr Lys Val Gln Gly Lys Asn Glu Asp Gln His Val Asp Val Lys
675 680 685

Ile Lys Asn Ala Thr Ile Ile Gln Tyr Gln Leu Lys Gly Leu Glu Pro
690 695 700

Glu Thr Ala Tyr Gln Val Asp Ile Phe Ala Glu Asn Asn Ile Gly Ser
705 710 715 720

Ser Asn Pro Ala Phe Ser His Glu Leu Val Thr Leu Pro Glu Ser Gln
725 730 735

Ala Pro Ala Asp Leu Gly Gly Gly Lys Met Leu Leu Ile Ala Ile Leu

740 745 750

Gly Ser Ala Gly Met Thr Cys Leu Thr Val Leu Leu Ala Phe Leu Ile
755 760 765

Ile Leu Gln Leu Lys Arg Ala Asn Val Gln Arg Arg Met Ala Gln Ala
770 775 780

Phe Gln Asn Val Arg Glu Glu Pro Ala Val Gln Phe Asn Ser Gly Thr
785 790 795 800

Leu Ala Leu Asn Arg Lys Val Lys Asn Asn Pro Asp Pro Thr Ile Tyr
805 810 815

Pro Val Leu Asp Trp Asn Asp Ile Lys Phe Gln Asp Val Ile Gly Glu
820 825 830

Gly Asn Phe Gly Gln Val Leu Lys Ala Arg Ile Lys Lys Asp Gly Leu
835 840 845

Arg Met Asp Ala Ala Ile Lys Arg Met Lys Glu Tyr Ala Ser Lys Asp
850 855 860

Asp His Arg Asp Phe Ala Gly Glu Leu Glu Val Leu Cys Lys Leu Gly
865 870 875 880

His His Pro Asn Ile Ile Asn Leu Leu Gly Ala Cys Glu His Arg Gly
885 890 895

Tyr Leu Tyr Leu Ala Ile Glu Tyr Ala Pro His Gly Asn Leu Leu Asp
900 905 910

Phe Leu Arg Lys Ser Arg Val Leu Glu Thr Asp Pro Ala Phe Ala Ile
915 920 925

Ala Asn Ser Thr Ala Ser Thr Leu Ser Ser Gln Gln Leu Leu His Phe
930 935 940

Ala Ala Asp Val Ala Arg Gly Met Asp Tyr Leu Ser Gln Lys Gln Phe
945 950 955 960

Ile His Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Gly Glu Asn Tyr
965 970 975

Val Ala Lys Ile Ala Asp Phe Gly Leu Ser Arg Gly Gln Glu Val Tyr
980 985 990

Val Lys Lys Thr Met Gly Arg Leu Pro Val Arg Trp Met Ala Ile Glu
995 1000 1005

Ser Leu Asn Tyr Ser Val Tyr Thr Thr Asn Ser Asp Val Trp Ser Tyr
1010 1015 1020

Gly Val Leu Leu Trp Glu Ile Val Ser Leu Gly Gly Thr Pro Tyr Cys
1025 1030 1035 1040

Gly Met Thr Cys Ala Glu Leu Tyr Glu Lys Leu Pro Gln Gly Tyr Arg
1045 1050 1055

Leu Glu Lys Pro Leu Asn Cys Asp Asp Glu Val Tyr Asp Leu Met Arg
1060 1065 1070

Gln Cys Trp Arg Glu Lys Pro Tyr Glu Arg Pro Ser Phe Ala Gln Ile
1075 1080 1085

Leu Val Ser Leu Asn Arg Met Leu Glu Glu Arg Lys Thr Tyr Val Asn
1090 1095 1100

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Thr Thr Leu Tyr Glu Lys Phe Thr Tyr Ala Gly Ile Asp Cys Ser Ala
1105                1110                1115                1120

Glu Glu Ala Ala
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1138 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Val Trp Arg Val Pro Pro Phe Leu Leu Pro Ile Leu Phe Leu Ala
1               5                  10                  15

Ser His Val Gly Ala Ala Val Asp Leu Thr Leu Leu Ala Asn Leu Arg
            20                  25                  30

Leu Thr Asp Pro Gln Arg Phe Phe Leu Thr Cys Val Ser Gly Glu Ala
        35                  40                  45

Gly Ala Gly Arg Gly Ser Asp Ala Trp Gly Pro Pro Leu Leu Leu Glu
    50                  55                  60

Lys Asp Asp Arg Ile Val Arg Thr Pro Pro Gly Pro Pro Leu Arg Leu
65                  70                  75                  80

Ala Arg Asn Gly Ser His Gln Val Thr Leu Arg Gly Phe Ser Lys Pro
                85                  90                  95

Ser Asp Leu Val Gly Val Phe Ser Cys Val Gly Gly Ala Gly Ala Arg
            100                 105                 110

Arg Thr Arg Val Ile Tyr Val His Asn Ser Pro Gly Ala His Leu Leu
        115                 120                 125

Pro Asp Lys Val Thr His Thr Val Asn Lys Gly Asp Thr Ala Val Leu
    130                 135                 140

Ser Ala Arg Val His Lys Glu Lys Gln Thr Asp Val Ile Trp Lys Ser
145                 150                 155                 160

Asn Gly Ser Tyr Phe Tyr Thr Leu Asp Trp His Glu Ala Gln Asp Gly
                165                 170                 175

Arg Phe Leu Leu Gln Leu Pro Asn Val Gln Pro Pro Ser Ser Gly Ile
            180                 185                 190

Tyr Ser Ala Thr Tyr Leu Glu Ala Ser Pro Leu Gly Ser Ala Phe Phe
        195                 200                 205

Arg Leu Ile Val Arg Gly Cys Gly Ala Gly Arg Trp Gly Pro Gly Cys
    210                 215                 220

Thr Lys Glu Cys Pro Gly Cys Leu His Gly Gly Val Cys His Asp His
225                 230                 235                 240
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Asp Gly Glu Cys Val Cys Pro Pro Gly Phe Thr Gly Thr Arg Cys Glu
                245                 250                 255

Gln Ala Cys Arg Glu Gly Arg Phe Gly Gln Ser Cys Gln Glu Gln Cys
            260                 265                 270

Pro Gly Ile Ser Gly Cys Arg Gly Leu Thr Phe Cys Leu Pro Asp Pro
        275                 280                 285

Tyr Gly Cys Ser Cys Gly Ser Gly Trp Arg Gly Ser Gln Cys Gln Glu
    290                 295                 300

Ala Cys Ala Pro Gly His Phe Gly Ala Asp Cys Arg Leu Gln Cys Gln
305                 310                 315                 320

Cys Gln Asn Gly Gly Thr Cys Asp Arg Phe Ser Gly Cys Val Cys Pro
                325                 330                 335

Ser Gly Trp His Gly Val His Cys Glu Lys Ser Asp Arg Ile Pro Gln
            340                 345                 350

Ile Leu Asn Met Ala Ser Glu Leu Glu Phe Asn Leu Glu Thr Met Pro
        355                 360                 365

Arg Ile Asn Cys Ala Ala Ala Gly Asn Pro Phe Pro Val Arg Gly Ser
    370                 375                 380

Ile Glu Leu Arg Lys Pro Asp Gly Thr Val Leu Leu Ser Thr Lys Ala
385                 390                 395                 400

Ile Val Glu Pro Glu Lys Thr Thr Ala Glu Phe Glu Val Pro Arg Leu
                405                 410                 415

Val Leu Ala Asp Ser Gly Phe Trp Glu Cys Arg Val Ser Thr Ser Gly
            420                 425                 430

Gly Gln Asp Ser Arg Arg Phe Lys Val Asn Val Lys Val Pro Pro Val
        435                 440                 445

Pro Leu Ala Ala Pro Arg Leu Leu Thr Lys Gln Ser Arg Gln Leu Val
    450                 455                 460

Val Ser Pro Leu Val Ser Phe Ser Gly Asp Gly Pro Ile Ser Thr Val
465                 470                 475                 480

Arg Leu His Tyr Arg Pro Gln Asp Ser Thr Met Asp Trp Ser Thr Ile
                485                 490                 495

Val Val Asp Pro Ser Glu Asn Val Thr Leu Met Asn Leu Arg Pro Lys
            500                 505                 510

Thr Gly Tyr Ser Val Arg Val Gln Leu Ser Arg Pro Gly Glu Gly Gly
        515                 520                 525

Glu Gly Ala Trp Gly Pro Pro Thr Leu Met Thr Thr Asp Cys Pro Glu
    530                 535                 540

Pro Leu Leu Gln Pro Trp Leu Glu Gly Trp His Val Glu Gly Thr Asp
545                 550                 555                 560

Arg Leu Arg Val Ser Trp Ser Leu Pro Leu Val Pro Gly Pro Leu Val
                565                 570                 575

Gly Asp Gly Phe Leu Leu Arg Leu Trp Asp Gly Thr Arg Gly Gln Glu
            580                 585                 590

Arg Arg Glu Asn Val Ser Ser Pro Gln Ala Arg Thr Ala Leu Leu Thr
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595 600 605

Gly Leu Thr Pro Gly Thr His Tyr Gln Leu Asp Val Gln Leu Tyr His
610 615 620

Cys Thr Leu Leu Gly Pro Ala Ser Pro Pro Ala His Val Leu Leu Pro
625 630 635 640

Pro Ser Gly Pro Pro Ala Pro Arg His Leu His Ala Gln Ala Leu Ser
645 650 655

Asp Ser Glu Ile Gln Leu Thr Trp Lys His Pro Glu Ala Leu Pro Gly
660 665 670

Pro Ile Ser Lys Tyr Val Val Glu Val Gln Val Ala Gly Gly Ala Gly
675 680 685

Asp Pro Leu Trp Ile Asp Val Asp Arg Pro Glu Glu Thr Ser Thr Ile
690 695 700

Ile Arg Gly Leu Asn Ala Ser Thr Arg Tyr Leu Phe Arg Met Arg Ala
705 710 715 720

Ser Ile Gln Gly Leu Gly Asp Trp Ser Asn Thr Val Glu Glu Ser Thr
725 730 735

Leu Gly Asn Gly Leu Gln Ala Glu Gly Pro Val Gln Glu Ser Arg Ala
740 745 750

Ala Glu Glu Gly Leu Asp Gln Gln Leu Ile Leu Ala Val Val Gly Ser
755 760 765

Val Ser Ala Thr Cys Leu Thr Ile Leu Ala Ala Leu Leu Thr Leu Val
770 775 780

Cys Ile Arg Arg Ser Cys Leu His Arg Arg Arg Thr Phe Thr Tyr Gln
785 790 795 800

Ser Gly Ser Gly Glu Glu Thr Ile Leu Gln Phe Ser Ser Gly Thr Leu
805 810 815

Thr Leu Thr Arg Arg Pro Lys Leu Gln Pro Glu Pro Leu Ser Tyr Pro
820 825 830

Val Leu Glu Trp Glu Asp Ile Thr Phe Glu Asp Leu Ile Gly Glu Gly
835 840 845

Asn Phe Gly Gln Val Ile Arg Ala Met Ile Lys Lys Asp Gly Leu Lys
850 855 860

Met Asn Ala Ala Ile Lys Met Leu Lys Glu Tyr Ala Ser Glu Asn Asp
865 870 875 880

His Arg Asp Phe Ala Gly Glu Leu Glu Val Leu Cys Lys Leu Gly His
885 890 895

His Pro Asn Ile Ile Asn Leu Leu Gly Ala Cys Lys Asn Arg Gly Tyr
900 905 910

Leu Tyr Ile Ala Ile Glu Tyr Ala Pro Tyr Gly Asn Leu Leu Asp Phe
915 920 925

Leu Arg Lys Ser Arg Val Leu Glu Thr Asp Pro Ala Phe Ala Arg Glu
930 935 940

His Gly Thr Ala Ser Thr Leu Ser Ser Arg Gln Leu Leu Arg Phe Ala
945 950 955 960

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Ser Asp Ala Ala Asn Gly Met Gln Tyr Leu Ser Glu Lys Gln Phe Ile
                965                 970                 975

His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Gly Glu Asn Leu Ala
            980                 985                 990

Ser Lys Ile Ala Asp Phe Gly Leu Ser Arg Gly Glu Glu Val Tyr Val
        995                 1000                1005

Lys Lys Thr Met Gly Arg Leu Pro Val Arg Trp Met Ala Ile Glu Ser
    1010                1015                1020

Leu Asn Tyr Ser Val Tyr Thr Thr Lys Ser Asp Val Trp Ser Phe Gly
1025                1030                1035                1040

Val Leu Leu Trp Glu Ile Val Ser Leu Gly Gly Thr Pro Tyr Cys Gly
                1045                1050                1055

Met Thr Cys Ala Glu Leu Tyr Glu Lys Leu Pro Gln Gly Tyr Arg Met
            1060                1065                1070

Glu Gln Pro Arg Asn Cys Asp Asp Glu Val Tyr Glu Leu Met Arg Gln
        1075                1080                1085

Cys Trp Arg Asp Arg Pro Tyr Glu Arg Pro Pro Phe Ala Gln Ile Ala
    1090                1095                1100

Leu Gln Leu Gly Arg Met Leu Glu Ala Arg Lys Ala Tyr Val Asn Met
1105                1110                1115                1120

Ser Leu Phe Glu Asn Phe Thr Tyr Ala Gly Ile Asp Ala Thr Ala Glu
                1125                1130                1135

Glu Ala
```

WHAT IS CLAIMED IS:

1. An isolated nucleotide sequence encoding a *tie*-2 protein.

2. A cDNA nucleotide sequence encoding a *tie*-2 protein.

3. A cDNA nucleotide sequence encoding a member of the MCK-10 family of proteins in which the nucleotide sequence encodes the amino acid sequence of FIG. 2 (SEQ. ID NO:), or which is capable of selectively hybridizing to the DNA sequence of FIG. 1 (SEQ. ID NO:).

4. A recombinant DNA vector containing a nucleotide sequence that encodes a *tie*-2 protein.

5. A recombinant DNA vector containing a nucleotide sequence that encodes a *tie*-2 fusion protein.

6. The recombinant DNA vector of Claim 4 in which the *tie*-2 nucleotide sequence is operatively associated with a regulatory sequence that controls the *tie*-2 gene expression in a host.

7. The recombinant DNA vector of Claim 5 in which the *tie*-2 fusion protein nucleotide sequence is operatively associated with a regulatory sequence that controls the *tie*-2 fusion protein gene expression in a host.

8. The DNA of Claim 2, 3, 4, 5, 6 or 7 in which the nucleotide sequence is capable of hybridizing under standard conditions, or which would be capable of hybridizing under standard conditions but for the

degeneracy of the genetic code to the DNA sequence of FIG. 1.

9. An engineered host cell that contains the recombinant DNA vector of Claims 4, 5, 6, or 7.

10. An engineered cell line that contains the recombinant DNA expression vector of Claim 6 and expresses *tie*-2.

11. An engineered cell line that contains the recombinant DNA expression vector of Claim 7 and expresses *tie*-2 fusion protein.

12. The engineered cell line of Claim 10 which expresses the *tie*-2 on the surface of the cell.

13. The engineered cell line of Claim 11 that expresses the *tie*-2 fusion protein on the surface of the cell.

14. A method for producing recombinant *tie*-2, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 9 and which expresses the *tie*-2; and

(b) recovering the *tie*-2 gene product from the cell culture.

15. A method for producing recombinant *tie*-2 fusion protein, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 11 and which expresses the *tie*-2 fusion protein; and

(b) recovering the *tie*-2 fusion protein from the cell culture.

16. An isolated recombinant *tie*-2 receptor protein.

17. A fusion protein comprising *tie*-2 linked to a heterologous protein or peptide sequence.

18. An oligonucleotide which encodes an antisense sequence complementary to the *tie*-2 nucleotide sequence, and which inhibits translation of the *tie*-2 gene in a cell.

19. The oligonucleotide of Claim 18 which is complementary to a nucleotide sequence encoding the amino terminal region of the *tie*-2.

20. A monoclonal antibody which immunospecifically binds to an epitope of the *tie*-2.

21. The monoclonal antibody of Claim 20 which competitively inhibits the binding of ligand to the *tie*-2.

22. The monoclonal antibody of Claim 20 which is linked to a cytotoxic agent.

23. The monoclonal antibody of Claim 20 which is linked to a radioisotope.

24. A method for screening and identifying antagonists of *tie*-2, comprising:

(a) contacting a cell line that expresses *tie*-2 with a test compound; and

(b) determining whether the test compound inhibits the binding of *tie*-2 ligand and the cellular effects of ligand binding on the cell line,

in which antagonists are identified as those compounds that inhibit both the binding and cellular effects of *tie*-2 ligand binding on the cell line.

25. A method for screening and identifying agonists of *tie*-2 ligand, comprising:

(a) contacting a cell line that expresses the *tie*-2 with a test compound in the presence and in the absence of ligand;

(b) determining whether, in the presence of ligand, the test compound inhibits the binding of ligand to the cell line and;

(c) determining whether, in the absence of the ligand, the test compound mimics the cellular effects of ligand on the cell line

in which agonists are identified as those test compounds that inhibit the binding but mimic the cellular effects of ligand on the cell line.

26. The method according to Claim 24 or 25 in which the cell line is a genetically engineered cell line.

27. The method according to Claim 24 or 25 in which the cell line endogenously expresses the *tie*-2.

28. A method for screening and identifying antagonists of *tie*-2 activity comprising:

(a) contacting *tie*-2 protein with a random peptide library such that *tie*-2 will recognize and bind to one or more peptide species within the library;

(b) isolating the *tie*-2/peptide combination;

(c) determining the sequence of the peptide isolated in step c; and

(d) determining whether the test compound inhibits the biological activity of *tie*-2.

in which agonists are identified as those peptides that inhibit the binding but mimic the cellular effects of *tie*-2.

29. A method for screening and identifying agonists of *tie*-2 comprising:

(a) contacting *tie*-2 protein with a random peptide library such that *tie*-2 will recognize and bind to one or more peptide species within the library;

(b) isolating the *tie*-2/peptide combination;

(c) determining the sequence of the peptide isolated in step c; and

(d) determining whether, in the absence of the *tie*-2 ligand, the peptide mimics the cellular effects of *tie*-2.

30. The method according to Claim 28 or 29 in which the *tie*-2 protein is genetically engineered.

31. A method of modulating the endogenous enzymatic activity of the tyrosine kinase *tie*-2 receptor in a mammal comprising administering to the mammal an effective amount of a ligand to the *tie*-2 receptor protein to modulate the enzymatic activity.

32. The method of Claim 31 in which the enzymatic activity of the receptor protein is decreased.

33. A recombinant vector containing a nucleotide sequence that encodes a truncated *tie*-2 which has

dominant-negative activity which inhibits the biological activity *tie*-2.

34. The recombinant vector of claim 33 in which the vector is a retrovirus vector.

35. An engineered cell line that contains the recombinant DNA vector of Claim 34 and expresses truncated *tie*-2.

36. An engineered cell line that contains the recombinant vector of Claim 35 and produces infectious retrovirus particles expressing truncated *tie*-2.

37. An isolated recombinant truncated *tie*-2 receptor protein which has dominant-negative activity which inhibits the biological activity of *tie*-2.

38. A method of modulating the biological activity of *tie*-2 in a mammal comprising administrating to the mammal an effective amount of truncated *tie*-2 receptor protein which inhibits the biological activity of *tie*-2 activation.

AGCAGGAGCC GGAGCAGGAG CAGAAGATAA GCCTTGGATG AAGGGCAAGA TGGATAGGGC TCGCTCTGCC 70

CCAAGCCCTG CTGATACCAA GTGCCTTTAA GATACAGCCT TTCCCATCCT AATCTGCAAA GGAAACAGGA 140

AAAAGGAACT TAACCCTCCC TGTGCTCAGA CAGAAATGAG ACTGTTACCG CCTGCTTCTG TGGTGTTTCT 210

CCTTGCCGCC AACTTGTAAA CAAGAGCGAG TGGACCATGC GAGCGGGAAG TCGCAAAGTT GTGAGTTGTT 280

GAAAGCTTCC CAGGGACTCA TGCTCATCTG TGGACGCTGG ATGGGGAGAT CTGGGGAAGT ATGGACTCTT 350

TAGCCGGCTT AGTTCTCTGT GGAGTCAGCT TGCTCCTTTA TGGAGTAGTA GAAGGTGCCA TGGACCTGAT 420

CTTGATCAAT TCCCTACCTC TTGTGTCTGA TGCCGAAACA TCCCTCACCT GCATTGCCTC TGGGTGGCAC 490

CCCCATGAGC CCATCACCAT AGGAAGGGAC TTTGAAGCCT TAATGAACCA GCACCAAGAT CCACTGGAGG 560

TTACTCAAGA TGTGACCAGA GAATGGGCGA AAAAAGTTGT TTGGAAGAGA GAAAAGGCCA GTAAGATTAA 630

TGGTGCTTAT TTCTGTGAAG GTCGAGTTCG AGGACAGGCT ATAAGGATAC GGACCATGAA GATGCGTCAA 700

CAAGCGTCCT TCCTACCTGC TACTTTAACT ATGACCGTGG ACAGGGGAGA TAATGTGAAC ATATCTTTCA 770

AAAAGGTGTT AATTAAAGAA GAAGATGCAG TGATTTACAA AAATGGCTCC TTCATCCACT CAGTGCCCCG 840

GCATGAAGTA CCTGATATTT TAGAAGTTCA CTTGCCGCAT GCTCAGCCCC AGGATGCTGG TGTGTACTCG 910

GCCAGGTACA TAGGAGGAAA CCTGTTCACC TCAGCCTTCA CCAGGCTGAT TGTTCGGAGA TGTGAAGCTC 980

AGAAGTGGGG GCCCGACTGT AGCCGTCCTT GTACTACTTG CAAGAACAAT GGAGTCTGCC ATGAAGATAC 1050

CGGGGAATGC ATTTGCCCTC CTGGGTTTAT GGGGAGAACA TGTGAGAAAG CTTGTGAGCC GCACACATTT 1120

GGCAGGACCT GTAAAGAAAG GTGTAGTGGA CCAGAAGGAT GCAAGTCTTA TGTGTTCTGT CTCCCAGACC 1190

CTTACGGGTG TTCCTGTGCC ACAGGCTGGA GGGGGTTGCA GTGCAATGAA GCATGCCCAT CTGGTTACTA 1260

CGGACCAGAC TGTAAGCTCA GGTGCCACTG TACCAATGAA GAGATATGTG ATCGGTTCCA AGGATGCCTC 1330

TGCTCTCAAG GATGGCAAGG GCTGCAGTGT GAGAAAGAAG GCAGGCCAAG GATGACTCCA CAGATAGAGG 1400

ATTTGCCAGA TCACATTGAA GTAAACAGTG GAAAATTTAA CCCCATCTGC AAAGCCTCTG GGTGGCCACT 1470

FIG.1A

ACCTACTAGT GAAGAAATGA CCCTAGTGAA GCCAGATGGG ACAGTGCTCC AACCAAATGA CTTCAACTAT 1540

ACAGATCGTT TCTCAGTGGC CATATTCACT GTCAACCGAG TCTTACCTCC TGACTCAGGA GTCTGGGTCT 1610

GCAGTGTGAA CACAGTGGCT GGGATGGTGG AAAAGCCTTT CAACATTTCC GTCAAAGTTC TTCCAGAGCC 1680

CCTGCACGCC CCAAATGTGA TTGACACTGG ACATAACTTT GCTATCATCA ATATCAGCTC TGAGCCTTAC 1750

TTTGGGGATG GACCCATCAA ATCCAAGAAG CTTTTCTATA AACCTGTCAA TCAGGCCTGG AAATACATTG 1820

AAGTGACGAA TGAGATTTTC ACTCTCAACT ACTTGGAGCC GCGGACTGAC TACGAGCTGT GTGTGCAGCT 1890

GGCCCGTCCT GGAGAGGGTG GAGAAGGGCA TCCTGGGCCT GTGAGACGAT TTACAACAGC GTCTATCGGA 1960

CTCCCTCCTC CAAGAGGTCT CAGTCTCCTG CCAAAAAGCC AGACAGCTCT AAATTTGACT TGGCAACCGA 2030

TATTTACAAA CTCAGAAGAT GAATTTTATG TGGAAGTCGA GAGGCGATCC CTGCAAACAA CAAGTGATCA 2100

GCAGAACATC AAAGTGCCTG GGAACCTGAC CTCGGTGCTA CTGAGCAACT TAGTCCCCAG GGAGCAGTAC 2170

ACAGTCCGAG CTAGAGTCAA CACCAAGGCG CAGGGGGAGT GGAGTGAAGA ACTCAGGGCC TGGACCCTTA 2240

GTGACATTCT CCCTCCTCAA CCAGAAAACA TCAAGATCTC CAACATCACT GACTCCACAG CTATGGTTTC 2310

TTGGACAATA GTGGATGGCT ATTCGATTTC TTCCATCATC ATCCGGTATA AGGTTCAGGG CAAAAATGAA 2380

GACCAGCACA TTGATGTGAA GATCAAGAAT GCTACCGTTA CTCAGTACCA GCTCAAGGGC CTAGAGCCAG 2450

AGACTACATA CCATGTGGAT ATTTTTGCTG AGAACAACAT AGGATCAAGC AACCCAGCCT TTTCTCATGA 2520

ACTGAGGACG CTTCCACATT CCCCAGCCTC TGCAGACCTC GGAGGGGGAA AGATGCTACT CATAGCCATC 2590

CTTGGGTCGG CTGGAATGAC TTGCATCACC GTGCTGTTGG CGTTTCTGAT TATGTTGCAA CTGAAGAGAG 2660

CAAATGTCCA AAGGAGAATG GCTCAGGCAT TCCAGAACGT GAGAGAAGAA CCAGCTGTGC AGTTTAACTC 2730

AGGAACTCTG GCCCTTAACA GGAAGGCCAA AAACAATCCG GATCCCACAA TTTATCCTGT GCTTGACTGG 2800

AATGACATCA AGTTTCAAGA CGTGATCGGA GAGGGCAACT TTGGCCAGGT TCTGAAGGCA CGCATCAAGA 2870

AGGATGGGTT ACGGATGGAT GCCGCCATCA AGAGGATGAA AGAGTATGCC TCCAAAGATG ATCACAGGGA 2940

CTTCGCAGGA GAACTGGAGG TTCTTTGTAA ACTTGGACAC CATCCAAACA TCATCAATCT CTTGGGAGCA 3010

FIG.1B

TGTGAACACC GAGGCTATTT GTACCTAGCT ATTGAGTATG CCCCGCATGG AAACCTCCTG GACTTCCTGC 3080

GTAAGAGCAG AGTGCTAGAG ACAGACCCTG CTTTTGCCAT CGCCAACAGT ACAGCTTCCA CACTGTCCTC 3150

CCAACAGCTT CTTCATTTTG CTGCAGATGT GGCCCGGGGG ATGGACTACT TGAGCCAGAA ACAGTTTATC 3220

CACAGGGACC TGGCTGCCAG AAACATTTTA GTTGGTGAAA ACTACATAGC CAAAATAGCA GATTTTGGAT 3290

TGTCACGAGG TCAAGAAGTG TATGTGAAAA AGACAATGGG AAGGCTCCCA GTGCGTTGGA TGGCAATCGA 3360

ATCACTGAAC TATAGTGTCT ATACAACCAA CAGTGATGTC TGGTCCTATG GTGTATTGCT CTGGGAGATT 3430

GTTAGCTTAG GAGGCACCCC CTACTGCGGC ATGACGTGCG CGGAGCTCTA TGAGAAGCTA CCCCAGGGCT 3500

ACAGGCTGGA GAAGCCCCTG AACTGTGATG ATGAGGTGTA TGATCTAATG AGACAGTGCT GGAGGGAGAA 3570

GCCTTATGAG AGACCATCAT TTGCCCAGAT ATTGGTGTCC TTAAACAGGA TGCTGGAAGA ACGGAAGACA 3640

TACGTGAACA CCACACTGTA TGAGAAGTTT ACCTATGCAG GAATTGACTG CTCTGCGGAA GAAGCAGCCT 3710

AGAGCAGAAC TCTTCATGTA CAACGGCCAT TTCTCCTCAC TGGCGCGAGA GCGCCTTGAC ACCTGTACCA 3780

AGCAAGCCAC CCACTGCCAA GAGATGTGAT ATATAAGTGT ATATATTGTG CTGTGTTTGG GACCCTCCTC 3850

ATACAGCTCG TGCGGATCTG CAGTGTGTTC TGACTCTAAT GTGACTGTAT ATACTGCTCG GAGTAAGAAT 3920

GTGCTAAGAT CAGAATGCCT GTTCGTGGTT TCATATAATA TATTTTTCTA AAAGCATAGA TTGCACAGGA 3990

AGGTATGAGT ACAAATACTG TAATGCATAA CTTGTTACTG TCCTAGATGT GTTTGATATT TTTCCTTTAC 4060

AACTGAATGC TATAAAAGTG TTTTGCTGTG TACACATAAG ATACTGTTCG TTAAAATAAG CATTCCCTTG 4130

ACAGCACAGG AAGAAAAGCG AGGGAAATGT ATGGATTATA TTAAATGTGG GTTACTACAC AAGAGGCCGA 4200

ACATTCCAAG TAGCAGAAGA GAGGGTCTCT CAACTCTGCT CCTCACCTGC AGAAGCCAGT TTGTTTGGCC 4270

ATGTGACAAT TGTCCTGTGT TTTTATAGCA CCCAAATCAT TCTAAAATAT GAACATCTAA AAACTTTGCT 4340

AGGAGACTAA GAACCTTTGG AGAGATAGAT ATAAGTACGG TCAAAAAACA AAACTGCGGG ACTTACATTT 4410

ATTTTCTATA GTAATCTGTT GTACATTTTA AGAAGTAAAA CTAGGAATTT AGGAGTGATG TGTGACATTT 4480

CTGACATGGA GTTACCATCC CCACATGTAT CACATACTGT CATATTCCCA CATGTATCAC ACATGTATTG 4550

TAAAATTTTG TAGTTTTGAT CACTTGTGAA TTTACTGTTG ATGTGGTAGC CACCTGCTGC AATGGTTCCT 4620

CTTGTAGGTG AATAAATGTC

FIG. 1C

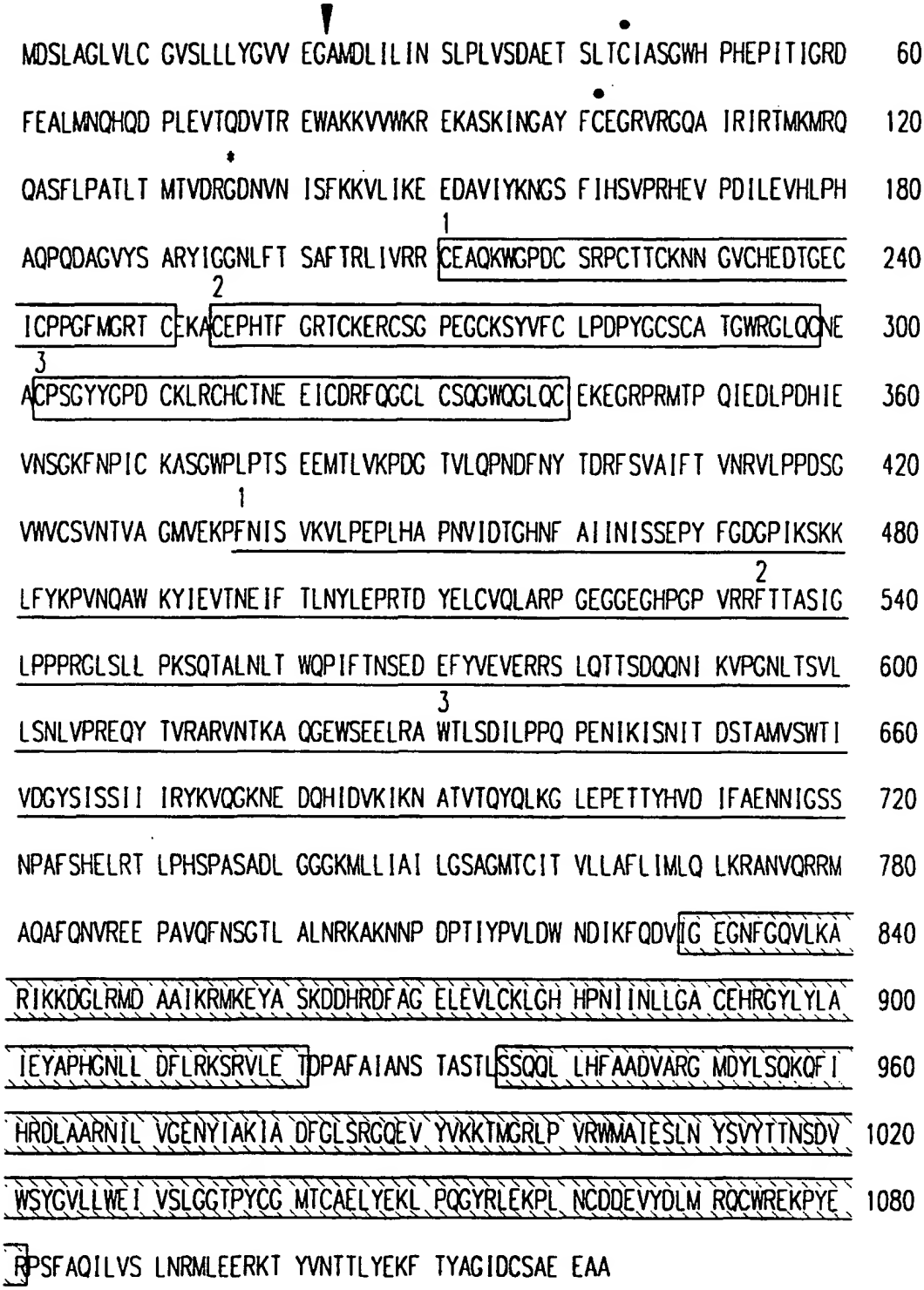

FIG.2A

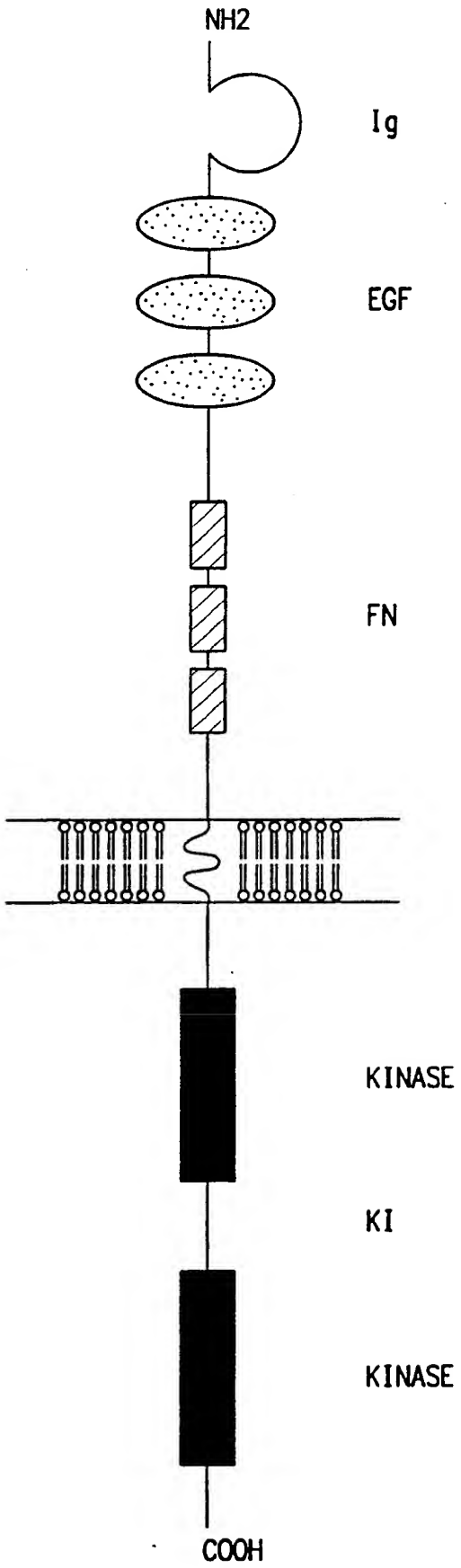

FIG.2B

```
      1                                                  50                                                 100
tie-2 .MDSLAGLVL CGVSLLLYGV VEGAMDLILI NSLPLVSDAE TSLTCIASGW HPHEPITIGR DFEALMNQHQ DPLEVTQDVT REWAKK.... .VVWKREKAS

TEK   .------S--- -------S-T --------- --------- --------- R--------- --------- --------- ------.... .---------
tie   MVWRVPPFL- ..PI-F-ASH -GA-V--T-L AN-R-TDPQR FF---V.--E AGAGRGSDAW GPPL-LEKDD RIVRTPPGPP LRL-RNGSHQ VTLRGFS-P-

      101                                                150                                                200
tie-2 KINGAYFCEG RVRGQAIRIR TMKMRQQASF LPATLTMTVD RGDNVNISFK KVLIKEEDAV IYKNGSFIHS VPRHEVPD.I LEVHLPHAQP QDAGVYSARY

TEK   --------- ----E----- --------- --------- K--VN----- --------- --------- ---------.- --------- ---------
tie   DLV-VFS-V- GAGARRT-VI YVHNSPG-HL --DKV-H--N K--TAVL-AR VHKE-QT-VI WKS---YFYT LDW--AQ-GR FLLQ--NV-- PSS-I---T-

      201                                                250                                                300
tie-2 IGGNLFTSAF TRLIVRRCEA QKWGPDCSRP CTTCKNNGVC HEDTGECICP PGFMGRTCEK ACEPHTFGRT CKERCSGPEG CKSYVFCLPD PYGCSCATGW

TEK   --------- --------- -----E-NHL --A-M------ --------- --------- ---L------ -------Q-- --------- ---------
tie   LEASPLG--- F------G-G- GR---G-TKE -PG-LHG--- -DHD---V-- ---T-TR--Q --REGR--QS -Q-Q-PGIS- -RGLT------ ------GS--

      301                                                350                                                400
tie-2 RGLQCNEACP SGYYGPDCKL RCHCTNEEIC DRFQGCLCSQ GWQGLQCEKE GRPRMTPQIE DLPDHIEVNS GKFNPI.CKA SGWPLPTSEE MTLVKPDGTV

TEK   K--------H P-F------- --S-N-G-M- ---------P --------R- -I------K-V --------- --------- -------N-- ---------
tie   --S--Q---A P-HF-A--R- Q-Q-Q-GGT- ---S--V-PS --H-VH---S D-...I---L NMASEL-F-L ETMPR-N-A- A-N-F-VRGS IE-R-------

      401                                                450                                                500
tie-2 LQPNDFNYTD RFSVAIFTVN RVLPPDSGVW VCSVNTVAGM VEKPFNISVK VLPEPLHAPN VIDTGHNFAI INISSEPYFG DGPIKSKKLF YKPVN..QAW

TEK   -H-K---H-- H-------IH -I-------- --------- --------- ---K--N--- ---------V --------- ---------L -----HYE--
tie   -LSTKAIVEP EKTT-EFE-P -LVLA---F- E-R-S-SG-Q DSRR-KVN-- -P-V--A--R LL.-KQSRQL VVSPLVSFS- ----STVR-H -R-QDSTMD-
```

FIG.3A

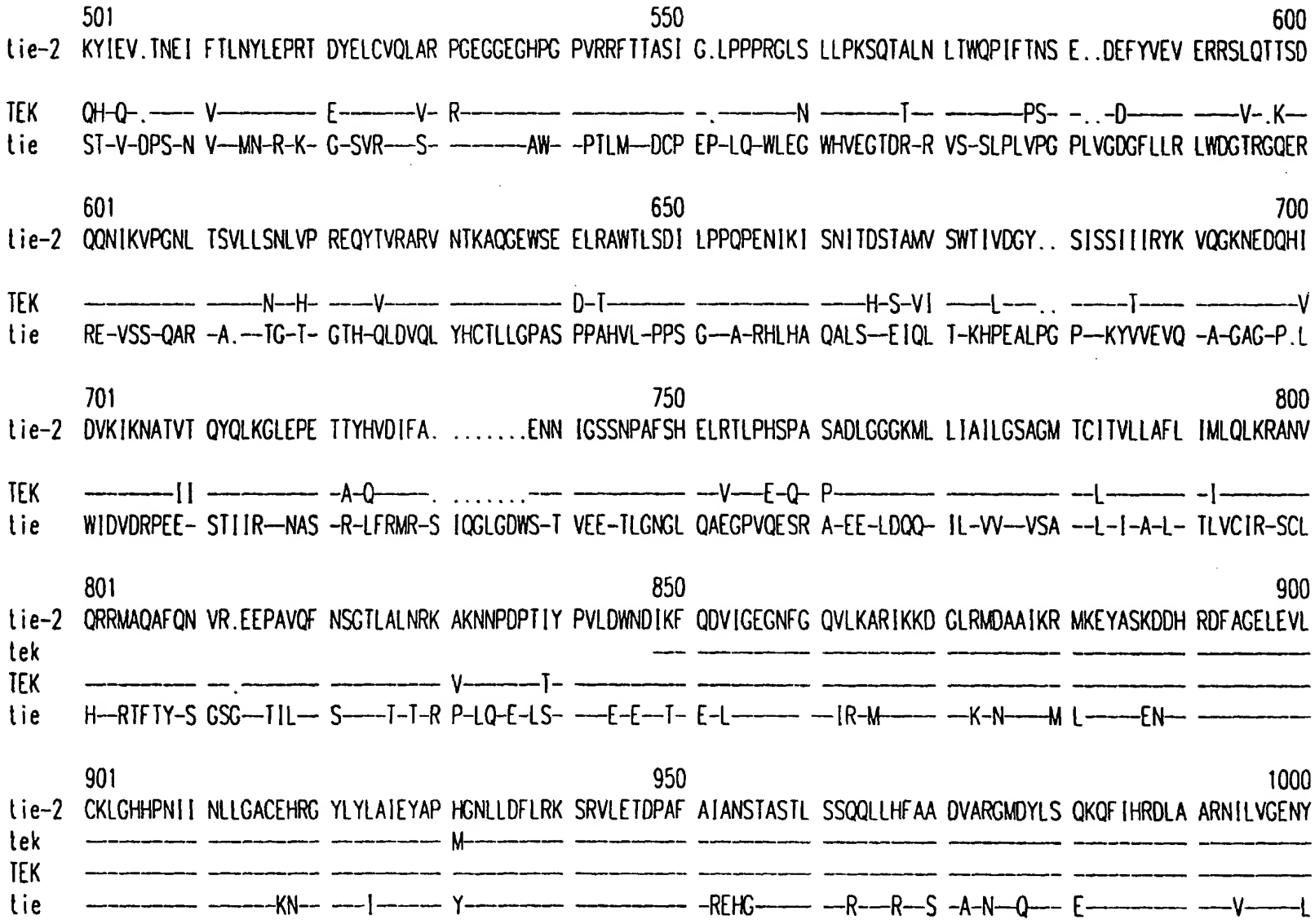

FIG.3B

```
         1001                                            1050                                                                 1100
lie-2    IAKIADFGLS RGQEVYVKKT MGRLPVRWMA IESLNYSVYT TNSDVWSYGV LLWEIVSLGG TPYCGMTCAE LYEKLPQGYR LEKPLNCDDE VYDLMRQCWR
tek      ---------- ---------I ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TEK      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
lie      AS-------- --E------- ---------- ---------- -K-------- ---------- ---------- -------AD- M-Q-R----- -E--------

         1101                                         1148
lie-2    EKPYERPSFA QILVSLNRML EERKTYVNTT LYEKFTYAGI DCSAEEAA
tek      ---------- ---------- ---------- ---------- --------
TEK      ---------- ---------- ---------- ---------- --------
lie      DR-----P-- --ALQ-G--- -A--A---MS -F-N------ -AT-----.
```

FIG.3C

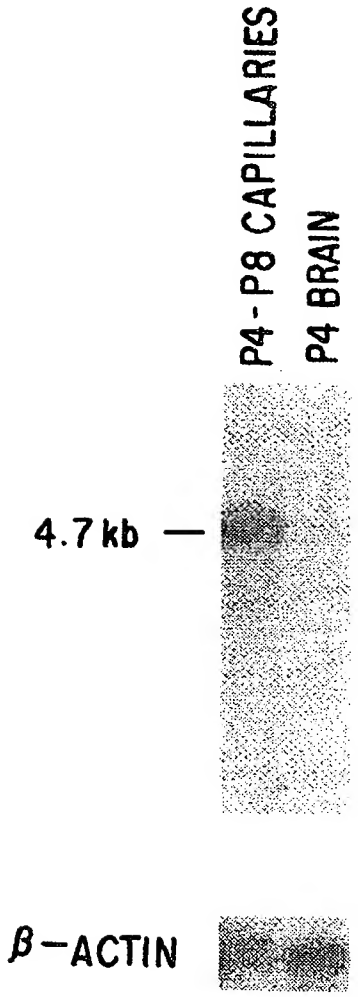

FIG. 4

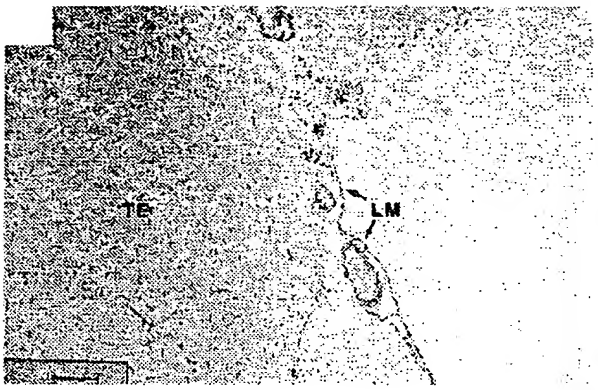

FIG.5A

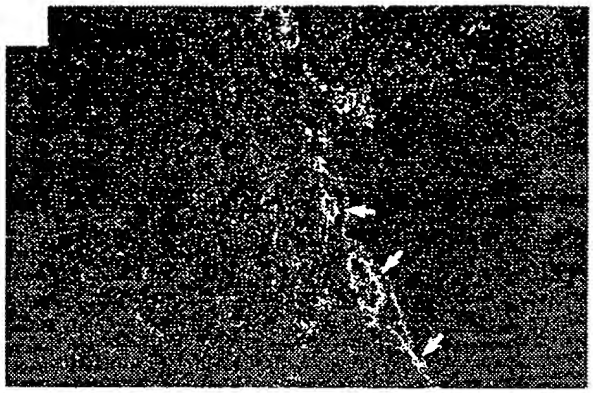

FIG.5B

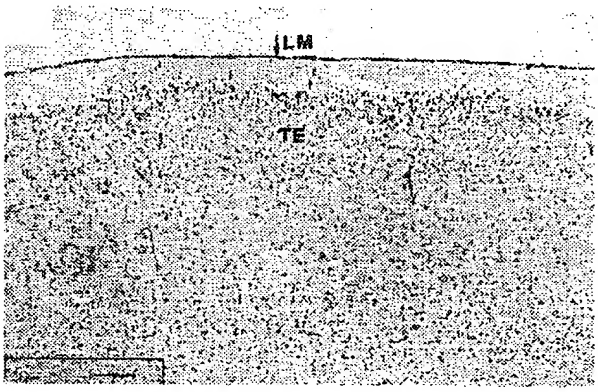

FIG.5C

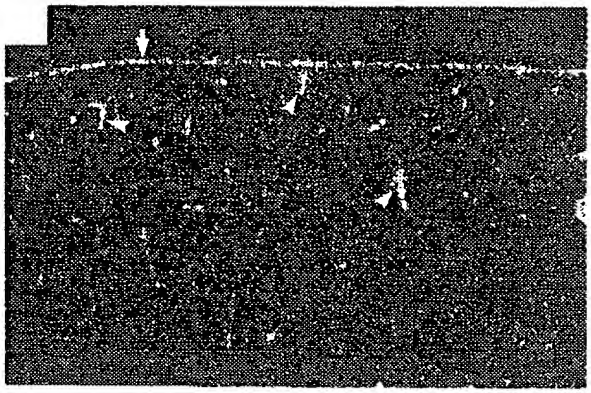

FIG.5D

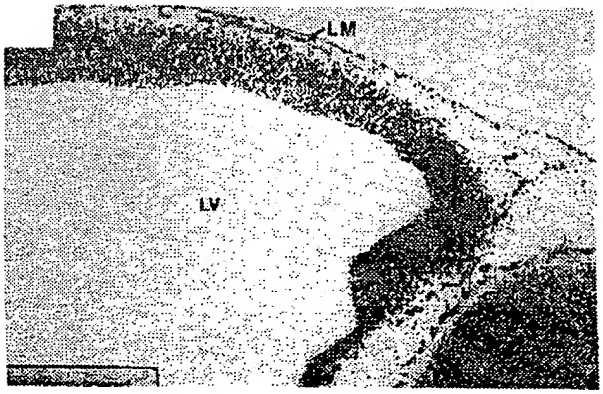

FIG.5E

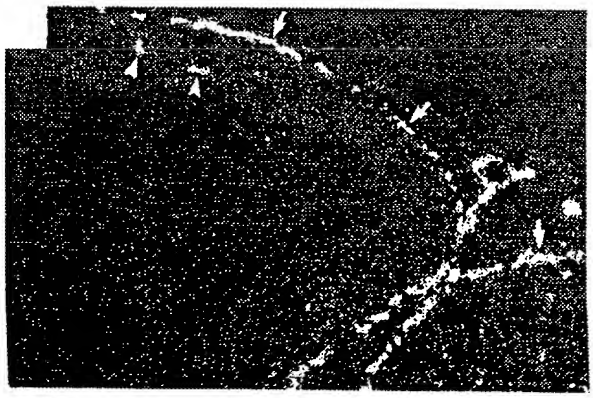

FIG.5F

FIG.5G

FIG.5H

FIG.6A

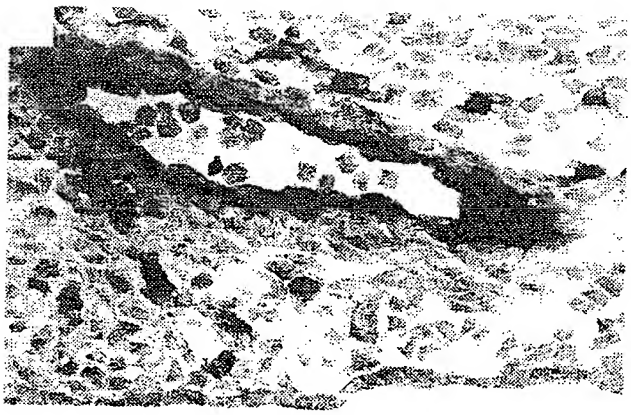

FIG.6B

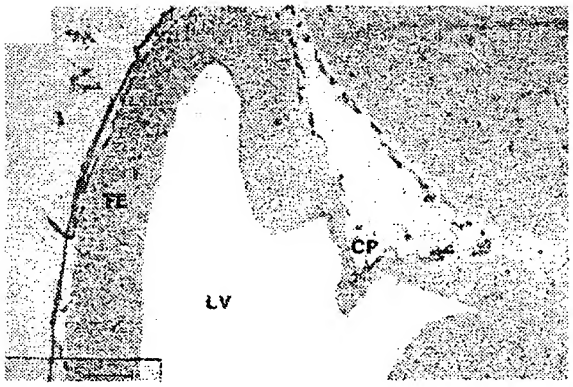

FIG.7A

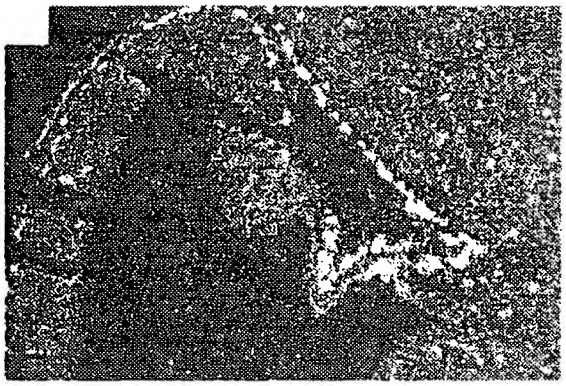

FIG.7B

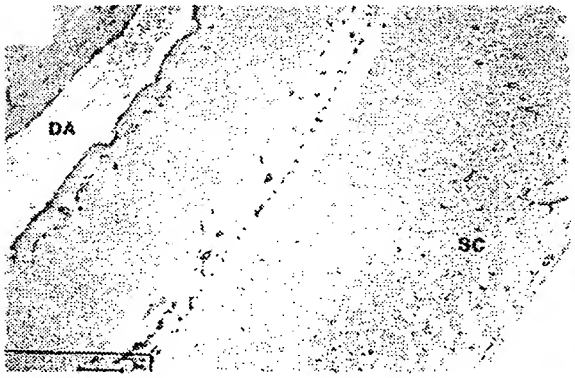

FIG.7C

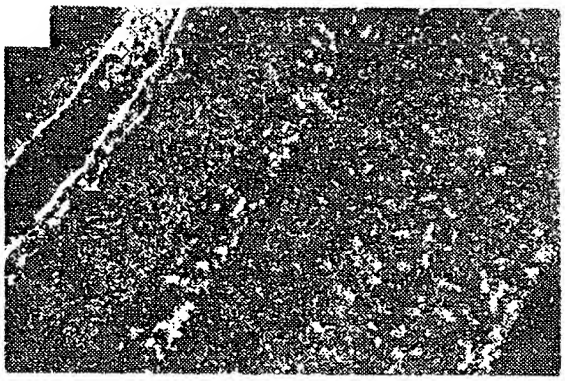

FIG.7D

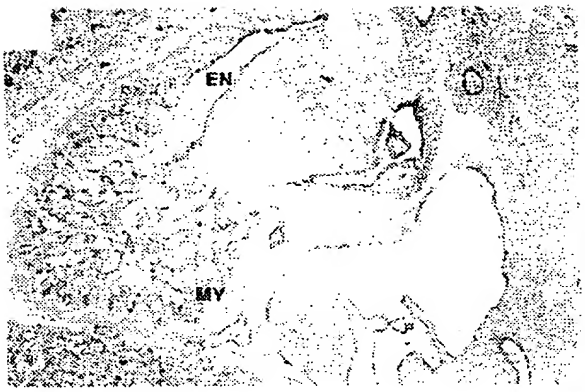

FIG.7E

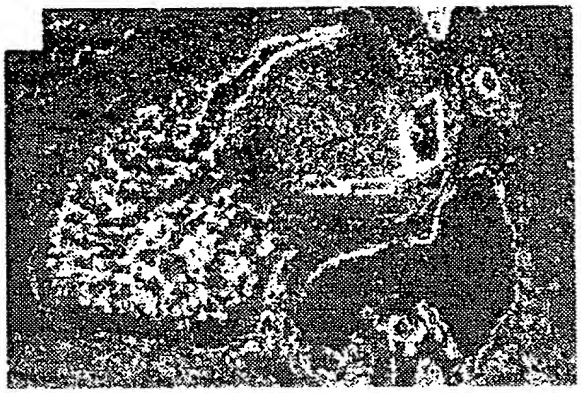

FIG.7F

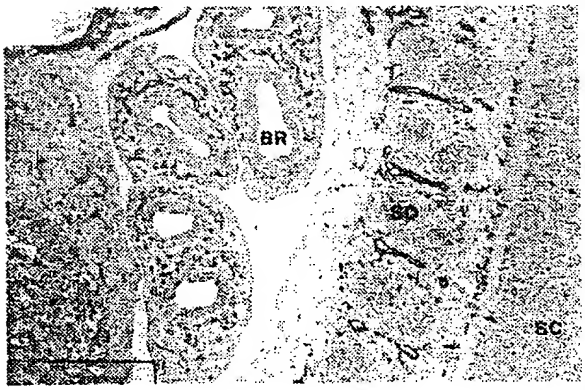

FIG.7G

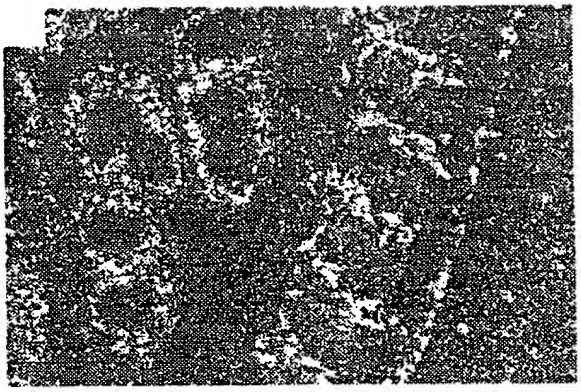

FIG.7H

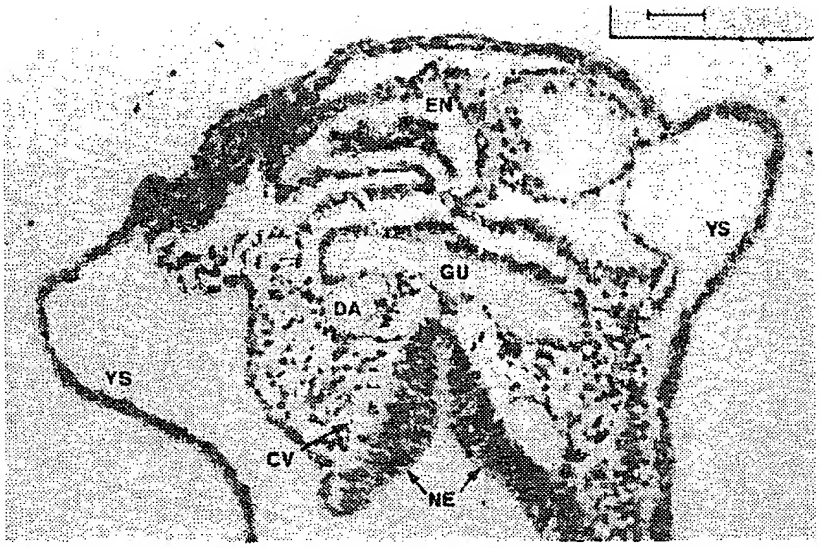

FIG.8A

FIG.8B

FIG.8C

FIG.8D

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C07K14/47 C07K14/515 C12N15/62 C12N5/16
C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEVELOPMENT, vol. 119, 1993 pages 957-968, H. SCHNÜRCH ET AL. 'Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage' *see the whole document* ---	1-38
X	GROWTH FACTORS, vol. 9, 1993 pages 99-105, A.S. RUNTING ET AL. 'tie-2, a putative protein tyrosine kinase from a new class of cell surface receptor' *see the whole document* --- -/--	1-38

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 1995	04.04.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Marie, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE, vol. 8, 1993 pages 1631-1637, P.C. MAISONPIERRE ET AL. 'Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family' *see the whole document* ---	1-38
X	PNAS, vol. 90, 1993 pages 9355-9358, T.N. SATO ET AL. 'tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system' *see the whole document* -----	1-38